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Physiology of Mycobacteria

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Abstract

Mycobacterium tuberculosis is a prototrophic, metabolically flexible bacterium that has achieved a spread in the human population that is unmatched by any other bacterial pathogen. The success of *M. tuberculosis* as a pathogen can be attributed to its extraordinary stealth and capacity to adapt to environmental changes throughout the course of infection. These changes include: nutrient deprivation, hypoxia, various exogenous stress conditions and, in the case of the pathogenic species, the intraphagosomal environment. Knowledge of the physiology of *M. tuberculosis* during this process has been limited by the slow growth of the bacterium in the laboratory and other technical problems such as cell aggregation. Advances in genomics and molecular methods to analyse the *M. tuberculosis* genome have revealed that adaptive changes are mediated by complex regulatory networks and signals, resulting in temporal gene expression coupled to metabolic and energetic changes. An important goal for bacterial physiologists will be to elucidate the physiology of *M. tuberculosis* during the transition between the diverse conditions encountered by *M. tuberculosis*. This review covers the growth of the mycobacterial cell and how environmental stimuli are sensed by this bacterium. Adaptation to different environments is described from the viewpoint of nutrient acquisition, energy generation and regulation. To gain quantitative understanding of mycobacterial physiology will require a systems biology approach and recent efforts in this area are discussed.

> "It is now 100 years since the first mycobacterium was isolated by Hansen (1874). Somewhat ironically, this was the leprosy bacillus, Mycobacterium leprae, which even today is still resisting all attempts to cultivate it in the laboratory. The tubercle bacillus, M. tuberculosis was not discovered until eight years later (Koch, 1882) and this has remained an object of intensive investigation ever since. The widespread interest in the mycobacteria of course stems from the diseases they cause and, lest it be imagined that tuberculosis is a disease which has now been largely conquered and that leprosy is of relatively rare occurrence, current estimates for the number of case of tuberculosis and leprosy in the world today are 20,000,000 and 11,000,000, respectively (Bechelli and Dominguez, 1972). The annual estimated mortality rate is equally dramatic, namely 3,000,000 (World Health Organization, 1974). Also causing unease is the continuing isolation from tubercular patients of strains already resistant to one or more chemotherapeutic agent".
>
> C. Ratledge (1976).

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1. INTRODUCTION

The growth and nutritional requirements of mycobacteria have been intensely studied since the discovery of *Mycobacterium tuberculosis* (Koch, 1882). These studies have resulted in an overwhelming body of literature on the physiology of mycobacterial metabolism in the years before the dawn of molecular biology (Edson, 1951; Ramakrishnan *et al.*, 1972; Ratledge, 1982). This review covers growth of the mycobacterial cell and how environmental stimuli are sensed by this bacterium. Adaptation to different environments is described from the viewpoint of nutrient acquisition, energy generation and regulation. We aim to present a clear view of current knowledge of the proliferation of mycobacteria *in vitro* in order to provide a better foundation for interpreting growth during stationary phase or growth under hypoxic conditions and growth *in vivo*. Other topics such as taxonomy, molecular methods for identifying species, epidemiology, and mechanisms of drug action are considered when they are appropriate to the narrative. One topic relevant to the main theme has not been discussed; namely detailed analysis of metabolism (e.g. fatty acids, amino acids) that have been reviewed previously by Ratledge (1976; 1982).

2. MYCOBACTERIA IN PERSPECTIVE

Our knowledge of bacterial growth has resulted from the study of relatively few strains that include *Escherichia coli* and *Bacillus subtilis*. Both *Mycobacterium leprae* (Hansen, 1874) and *Mycobacterium tuberculosis* (Koch, 1882) are amongst the first microbes to have been identified and yet our knowledge of their physiological properties has lagged behind our insights into bacterial physiology in general. Mycobacterial strains are not always easy to grow in the laboratory. For example, *M. leprae* has been harvested from infected laboratory animals and has never been grown *in vitro*. *Mycobacterium genavense* was identified by molecular techniques and has yet to be grown in culture (Böttger, *et al.*, 1992; Coyle, *et al.*, 1992). Other species such as *M. ulcerans* and *M. paratuberculosis* produce visible growth on a solid medium only after a month or more in culture (Wayne and Kubica, 1986). Typically, slow growing mycobacteria produce visible colonies on a solid medium within 10 – 28 days. Apart from slow growth, other difficulties in studying mycobacteria include the hazards of handling pathogens, the tendency of cells to clump, and the resistance of cells to lysis.

2.1. *Mycobacterium* and its close relatives

Mycobacteria are Gram-positive, acid-fast and (G + C) rich (62 – 70%); they are aerobic and rod shaped and mycolic acids are components of their cell walls (for review see Wayne and Kubica, 1986). Other bacteria including *Nocardia*, *Rhodococcus* and *Corynebacterium* have related properties (see Table 1). The genera *Corynebacterium*, *Nocardia* and *Mycobacterium* form a well defined sub -group (CMN) of actinobacteria (Embley and Stackebrandt, 1994; Ventura *et al.*, 2007). Other genera that synthesize mycolic acids include *Dietza*, *Gordona. Skermania*, *Tsukamurella*, *Turicella* and *Williamsia* (Gurtler *et al.*, 2004).

2.2. Mycobacterium

The genus *Mycobacterium* is now known to comprise more than one hundred species which are all closely related as judged by comparison of their 16S rRNA sequences (Tortoli, 2006). This rapid expansion of the genus since 1990 is based on the invention of the Polymerase Chain Reaction (PCR) for the amplification of DNA sequences (Saiki *et al.*, 1985) and the recognition of the value of 16S rRNA sequences as phylogenetic markers (Woese, 1987). These methods were first applied to mycobacteria less than twenty years ago (Stahl and Urbance, 1990; Rogall *et al.*, 1990a; 1990b). Since then, mycobacteria have been found to be widely distributed in the environment. Mycobacteria are opportunistic pathogens, especially in a clinical environment, and their detection is important to treatment of the

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infections they cause. Mycobacteria also have possible industrial appplications. *M. austroafricanuum* is able to metabolize methyl tert-butyl ether which is often added to gasoline and could be used to treat contaminated ground water (Maciel *et al.*, 2008).

The earliest studies of mycobacterial genes concerned the rRNA operons (Cox and Katoch, 1986; Bercovier *et al.*, 1986). After a little more than a decade later, the genome sequences of *M. tuberculosis* H37Rv (Cole *et al.*, 1998) and *M. leprae* (Cole *et al.*, 2001) were published. Knowledge of these sequences has facilitated very rapid progress in mycobacterial research. The number of published genome sequences has now reached seventeen.

3. PROPERTIES OF MYCOBACTERIAL CELLS

3.1. Population-average cells

Measurements based on cell cultures provide information about population-average cells (Schaechter *et al.*, 1958) and the picture that emerges is that of a virtual cell. A bacterial culture comprises cells of all ages ranging from new born cells of age $a = 0$ to cells about to divide (age $a = 1$). The age range of cells comprising a culture remains constant as the number of cells increase (Powell, 1956) during both exponential growth in batch culture and growth in continuous culture (a chemostat). Thus, the virtual cell is representative of the entire age range. DNA, RNA and protein fractions isolated from cell cultures provide information about population-average cells. Suppose, that D, R, P and N_c refer to the mass of DNA, RNA and protein respectively per ml of culture and N_c is the number of cells per ml of culture then $\boldsymbol{m}_{\mathrm{DNA(av)}}$, $\boldsymbol{m}_{\mathrm{RNA(av)}}$ and $\boldsymbol{m}_{\mathrm{p(av)}}$ which are respectively the masses of DNA, RNA and protein per population-average cell are defined in equation (1) – (3).

$$m_{\mathrm{DNA(av)}} = D/N_c \quad (1)$$

$$m_{\mathrm{RNA(av)}} = R/N_c \quad (2)$$

$$m_{p(\mathrm{av})} = P/N_c \quad (3)$$

Furthermore, the synthesis rates such as $\omega_{\mathrm{p(av)}}$ the specific protein synthesis rate [see equation (4)].

$$\omega_{p(\mathrm{av})} = \mu \bullet m_{p(\mathrm{av})} \quad (4)$$

and $\omega_{\mathrm{RNA(av)}}$ the specific RNA synthesis rate [see equation (5)]

$$\omega_{\mathrm{RNA(av)}} = \mu \bullet m_{\mathrm{RNA(av)}} \quad (5)$$

describe the population-average cell.

A schematic view of a population-average cell of *M. tuberculosis* was devised to provide an instantaneous view of the macromolecular composition ($\boldsymbol{m}_{\mathbf{DNA(av)}}$, $\boldsymbol{m}_{\mathrm{RNA(av)}}$ and $\boldsymbol{m}_{\mathrm{p(av)}}$) and of protein synthesis and other features (Cox and Cook, 2007).

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3.2. Cell size and shape

Mycobacterial cells are irregular rods 0.3–0.5 μm in diameter and of variable length (Wayne and Kubica, 1986). The images of cells of *M. smegmatis* shown in Fig. 1 show that the increase in cell size is reflected in an increase in length rather than the diameter of the rods (compare left and right panels). For example, any changes in the diameter are small whereas length ranges from 1.5 μm–4.0 μm according to the period of culture. This mode of cell growth provides the cell with a high ratio of surface area to mass, which is favourable for cells that are relatively impervious to aqueous solvents; that is, the number of porin channels per unit surface area is low compared with *E. coli*. It has been shown that nutrients are taken up by the cell by passive diffusion through the permeability barrier and then actively transported across the cytoplasmic membrane (Nikaido and Rosenberg, 1981; Stephan *et al.,* 2005; please see section 8). Hence, *in vitro* the rate of passive diffusion can be increased by increasing the concentration of nutrients in the growth medium and hence allow the optimal growth rate to be attained. It is not known if this situation (occasions of 'feast') is often encountered in the environment.

3.3. Macromolecular composition

Measurements of amounts of DNA, RNA and proteins per population-average cell at 'defined' specific growth rates provide valuable insights into cell metabolism. Ninety per cent of the energy needed for macromolecular synthesis is devoted to protein synthesis, which accounts for approximately one half of the energy needed for cell growth. The incorporation of each amino acid into a polypeptide chain is achieved at the expense of four high-energy phosphate bonds (for review see Cox and Arnstein, 2007). Cell growth requires protein synthesis, which, in turn requires ribosome synthesis. These aspects of cell proliferation are reflected in the ratios DNA:RNA : protein. However, few data are available for mycobacteria largely because of the difficulties encountered in lysing cells. Available data for *M. tuberculosis* H37Rv and *M. bovis* BCG are summarized in Table 2. There is little agreement between the three sets of data. The observed differences are probably too large to be accounted for by differences in growth conditions. Technical difficulties are more likely to be responsible for the wide range of reported values. For example, Youmans and Youmans (1968) used the orcinal method to measure RNA content and this reagent also reacts with polysaccharides. Beste *et al.* (2005) used different methods to lyse cells for measurements of DNA, RNA and protein and failed to use a secondary standard to measure the extents of cell lysis. The data reported by Winder and Rooney (1970) was shown to be consistent with an equation derived by Bremer (1975) relating the growth rate with parameters affecting the rates of synthesis of ribosomes and RNA polymerase in bacteria (Colston and Cox, 1999). Thus, there is a dearth of information about the DNA, RNA and protein contents of mycobacteria.

3.4. Does genome size influence the specific growth rate?

The genome sizes of mycobacterial species obtained by DNA sequencing range from 3,268,203 base-pairs (*M. leprae*) to 6,988,209 base-pairs (*M. smegmatis* mc ^{2}155). Optimum specific growth rates have been reported for *M. tuberculosis* H37Rv (Wayne, 1994), *M. marinum* (Clark and Shepard, 1963) and *M. smegmatis* mc ^{2}155 (Sander *et al.*, 1996). The range in genome sizes poses the question; 'How can specific growth rates be related to genome sizes?' This problem was approached in the following way. *E. coli* was chosen as the reference species (The Eco_model) because it is widely studied and an extensive data base is available (see Bremer and Dennis, 1996). The properties of *E. coli* B/r grown at 37°C in a minimal salts medium with succinate as the carbon source were used as its reference state because, under these conditions of growth, the genome is replicated once only during the cell division cycle (Bremer and Dennis, 1996). Previously, it was found that equations developed to relate the specific growth rates of *E. coli* B/r with its macromolecular

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composition (Bremer, 1975) also applied to *M. bovis* BCG (Colston and Cox, 1999). Quantitative relationships relating specific growth rates and macromolecular composition of *M. tuberculosis*, *Streptomyces coelicolor* A3(2) and *E. coli* B/r were used to develop an instantaneous view of macromolecular synthesis (Cox, 2004). Thus, *E. coli* grown in succinate medium is a suitable model for the other strains considered in this review; namely mycobacteria, corynebacteria and streptomyces, which also replicate their genomes once only during the growth cycle. The model is based on a generation time t_D of 1.67 h, an interval of t_{G1}= 0.05 h between cell division and the start of DNA replication (S-phase) which continues for τ_g= 1.17 h (the genome is replicated at a rate of ε_{DNA}= 2.08 × 10^6 base -pairs h^{-1} per replication fork); S-phase is followed by the period t_{G2}= 0.5 h before cell division takes place. It is supposed that if the species under scrutiny were as metabolically active as *E. coli* B/r growing in succinate medium at 37°C then t_{G1}, t_{G2} and ε_{DNA} would have the same values as the *E. coli* B/r model. However, in this model τ_g the period of DNA synthesis is directly proportional to l_g base-pairs, the genome size of the species of interest. The generation time t_D* of the Eco_model is given by equation (6) where *E. coli* values are shown by the superscript # sign.

$$t_D{}^* = t_{G1}{}^{\#} + (l_g / 2\varepsilon_{DNA}{}^{\#}) + t_{G2}{}^{\#} \quad (6)$$

Hence, μ* the specific growth rate of the Eco_model may be evaluated. The Eco_index, which is the ratio of the observed and Eco_model derived specific growth rates, provides a measure of the relative metabolic activities of the subject species growing optimally in an appropriate medium and *E. coli* B/r growing in succinate medium at 37°C.

The model was applied to three mycobacterial species, which have well characterized optimal specific growth rates, and two other species, namely *Streptomyces coelicolor* A3(2) and *Corynebacterium glutamicum* ATCC 13032 that have genomes of 8,667,507 and 3,309,401 base-pairs, respectively. The results are shown in Table 3. The Eco_index was found to vary from 0.1 (*M. tuberculosis* H37Rv) to 1.15 (*S. coelicolor* A3(2)). Three species grow optimally at 30°C; namely *M. marinum* strain M, *C. glutamicum*, and *S. coelicolor* A3(2). No correction was applied to allow for growth at different temperatures. The results indicate that both *M. smegmatis* mc^2155 and *M. marinum* strain M have specific growth rates that are close (74% and 80%, respectively) to the value predicted for the Eco_model. The model for *M. smegmatis* mc^2155 is based on a DNA replication rate of ε_{DNA}= 2.08 × 10^6 base -pairs h^{-1} per replication fork. The value of ε_{DNA}= 2.0 × 10^6 base-pairs h^{-1} per replication fork can be deduced from an observed S-phase of 1.75 h (Hiriyanna and Ramakrishnan, 1986), which is very close to the rate observed for *E. coli* B/r grown in succinate medium.

In contrast, the Eco_index of 0.1 derived for *M. tuberculosis* H37Rv indicates a metabolic activity of 10% of the *E. coli* B/r model. This expectation is accord with the observed value of ε_{DNA} = 0.21 × 10^6 base -pairs h^{-1} per replication fork deduced from the reported S-phase of 10.33 h (Hiriyanna and Ramakrishnan, 1986), which is close to one tenth of the rate observed for *E. coli* B/r. To a first approximation, the Eco_index reflects the relative rates of DNA replication, which provides a guide to the relative metabolic activities of the species concerned. If an allowance was made for growth at 30°C, then the Eco_index of *M. marinum*, *C. glutamicum*, and *S. coelicolor* A3(2) would be closer to or even exceed 1.0.

3.5. Mycobacterial *rrn* operons

Classical pathogenic slow growers such as *M. tuberculosis* were shown to have a single *rrn* operon per genome whereas classical fast growers such as *M. smegmatis* and *M. phlei* were found to have two (Bercovier *et al.*, 1986; Domenech *et al.*, 1994). The generalization that

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mycobacteria have either one or two *rrn* operons per genome has proved to be correct. All the mycobacteria studied to date were found to have one operon (*rrnA*) located downstream from *murA* (Gonzalez-y-Merchand *et al*., 1996, 1997; Helguera-Reppetto *et al*., 2004; Rivera-Guttiérez *et al*., 2003): the second operon (*rrnB*), if present, was found downstream from *tyrS* (Gonzalez -y-Merchand *et al*., 1996; Menendez *et al*., 2002; Predich *et al*., 1995). A phylogenetic tree based on 16S rRNA sequences was related to the numbers of *rrn* operons per genome. The combined data supports the view that the ancestral *Mycobacterium* possessed one *rrnA* and one *rrnB* operon and that on two separate occasions an *rrnB* operon was lost giving rise to two clusters of mycobacteria possessing a single *rrn* operon (*rrnA*) per genome (Stadthagen-Gomez *et al*., 2008). One cluster includes the classical pathogens *M. leprae* and *M. tuberculosis* and the other includes *M. abscessus* and *M. chelonae*.

3.5.1. The number of *rrn* operons per genome is not a reliable guide to growth rate—The number of operons per genome was found to be an unreliable guide to growth rate. For example, *M. chelonae* and *M. abscessus* are classified as fast growers and yet each has single *rrn*(*rrnA*) operon per genome (Domenech *et al*., 1994). In contrast, two slow growers, *M. terrae* (Ninet *et al*., 1996) and *M. celatum* (Reischl *et al*., 1998) were each found to have two *rrn* operons per genome. The fish pathogen *M. marinum* which has a single *rrn*(*rrnA*) operon per genome is classified as a slow grower that is closely related to *M. tuberculosis* (Goodfellow and Magee, 1998; Devulder *et al*., 2005) grows optimally at 30°C with a generation time of 4 h (Clark and Shepard, 1963; Cosma *et al*., 2003) compared with a generation time of 16 h or more at 37°C for *M. tuberculosis*. The explanation for the poor correlation found between the number of *rrn* operons and specific growth rate was provided by Sander *et al*. (1996) who showed that the specific growth rate of *M. smegmatis* mc^2155 was unaltered by inactivation of either one or the other of its two *rrn* operons: in other words, the second *rrn* operon is redundant. The replication of *rrn* operons is discussed in the following section.

4. DNA REPLICATION

Our knowledge of the replication of the genomes of mycobacteria is limited to a single study of the duration of S-phases of *M. smegmatis* and *M. tuberculosis* H37Rv (Hiriyanna and Ramakrishnam, 1986). However, it is assumed that the current view of the replication of a circular bacterial genome applies. Replication is initiated at a single site, *oriC,* and proceeds in both clockwise and counterclockwise directions until the terminus (*ter*) is reached (Prescott and Kuempel, 1972; Higgins, 2007). The genome is replicated once only during the growth cycle (see for example Boye *et al*., 2000; Zakrzewska-Czerwinska *et al*., 2007). DNA replication occurs during S-phase at an average rate of Σ_{DNA}base-pairs h^{-1} per replication fork. S-phase is preceded by G1-phase which is needed to prepare the enzymes for DNA synthesis and is followed by G2-phase during which the newly synthesized genomes are segregated and septum formation takes place.

4.1. Replication of individual ORFs

The replication of a particular ORF occupies only a small fraction of S-phase. An ORF ($ORF_{(0)}$) located close to *oriC* is replicated at the beginning of S-phase when two copies become available for the rest of the cell's lifetime. In principle it can benefit the cell if an ORF, whose gene product is in high demand, is located near to *oriC*. Then the newly replicated ORF becomes available at the earliest opportunity; namely the end of G1-phase. In contrast, an ORF ($ORF_{(t)}$) located near to *ter* provides the cell with a newly replicated copy for no longer than the G2-period.

The time during S-phase when $ORF_{(i)}$ is replicated depends on the position of $ORF_{(i)}$ within the genome. The positions of *rrn* operons are variable and the replication coefficient

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facilitates comparisons between mycobacteria. The replication coefficient γ is defined as the shortest distance (base pairs) of an $ORF_{(i)}$ from *oriC* measured either clockwise or counter clockwise divided by one half the length (base-pairs) of the genome. The replication coefficients of mycobacterial *rrn* operons are greater than 0.5 revealing that they are replicated in the second half of S-phase. In contrast, the replication coefficients of the ten *rrn* operons of *Bacillus subtilis* subsp *subtilis* are close to zero showing they are replicated very early in S-phase (Kunst *et al.,* 1997). The replication coefficient $\gamma_{ORF(i)}$ is related to the time $\tau_{ORF(i)}$ when $ORF_{(i)}$ is replicated in S-phase. In turn, $\tau_{ORF(i)}$ plus the G1 period is equal to the time $t_{ORF(i)}$ during the cell division cycle when $ORF_{(i)}$is replicated. The age $a_{(i)\bullet R}$ of a cell when $ORF_{(i)}$ is replicated is obtained by dividing $t_{ORF(i)}$ by t_D the generation time Once $a_{(i)\bullet R}$ is known the number of copies of $ORF_{(i)}$ per population-average cell can be calculated (see Appendix A). The limited data available for *M. tuberculosis* H37Rv shows that the *rrnA* operon ($\gamma = 0.67$) is replicated in the second half of S-phase; leading to 1.36 copies of *rrnA* per population -average cell. In contrast, $ORF_{(0}$ located close to *oriC* is present as 1.72 copies per population-average cell, whereas there are 1.20 copies of $ORF_{(t)}$ located near to *ter*.

The available data for *M. smegmatis* mc^2155 show that this bacterium can grow at its optimum rate with a single *rrnB* operon; this operon, which has a replication coefficient of 0.90, is replicated late in S-phase. Hence, this mutant has 1.18 copies of *rrnB* per population -average cell. Thus, a single *rrn* operon that is replicated late in S-phase is sufficient to support mycobacterial growth of 3 h generation time, a rate that is 74% of the Eco_model (see Table 4). Moreover, when the *M. tuberculosis rrnA* operon was expressed in *M. smegmatis* it was competitive with the *rrnA* and *rrnB* operons of the host (Verma *et al.*, 1999). By analogy, it is safe to conclude that the single *rrnA* operon of *M. tuberculosis*, which is present at 1.36 copies per population-average cell (see above) is also sufficient to support a generation time of 3 h. In other words, the possession of a single *rrn* operon per genome is not related to the slow growth of the tubercle bacillus. This view is reinforced by the demonstration that *E. coli* can grow with a specific growth rate of $\mu = 0.6\ h^{-1}$ even after inactivation of six of its seven *rrn* operons or even after inactivation of all seven operons with a functional operon provided by a plasmid (Asai *et al.*, 1999). Fluorescence spectroscopy revealed that deletion of six or seven *rrn* operons changed the size and shape of the cells from short and fat to long and thin like mycobacteria.

5. MYCOBACTERIAL RIBOSOMES

Ribosomes of the *M. tuberculosis* complex were shown to have sedimentation properties similar to those of ribosomes of *E. coli* (Worcel *et al.,* 1968; Trnka *et al.*, 1968), with 16S, 23S and 5S rRNA components (Worcel *et al.,* 1968). The ribosome fraction was shown to be active in cell-free protein synthesis (Shaila *et al.*, 1973).

5.1. Components of mycobacterial ribosomes

The RNA (rRNA) moiety of ribosomes is encoded by classical rRNA (*rrn*) operons in the order 5′ 16S rRNA, 23S rRNA and 5S rRNA 3′ (Suzuki *et al.*, 1988; Liesack *et al.*, 1990, 1991; Kempsell *et al.*, 1992). The information available for ribosomal proteins (r-proteins) is largely derived from genomic sequences. For example, r-proteins of *M. tuberculosis* are orthologues of *E. coli* r-proteins. However, four r-proteins: namely, S14, S18, L28 and L33 (see Table 5) are each encoded by two non identical genes. Not all of the available mycobacterial genomic sequences were found to have these extra genes. When present, the four genes were located as a group encoding r-proteins in the order 5′ L28, L33, S14, S18 3′. This cluster of genes is absent from *M. leprae, Mycobacterium species* JLS, *Mycobacterium species* KMS and *Mycobacterium* species *MCS.* The extra set of genes encode four proteins which have different amino acid sequences from their standard

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orthologues; for example, two proteins with non-identical amino acid sequences are identified as orthologues of *E. coli* L28. The non-identical orthologues of each of the four r-proteins raises the question of the homogeneity of the ribosome fraction. If ribosomes are heterogeneous with respect to their complement of r-proteins is ribosome function affected? Microarray measurements reveal that the additional set of r-proteins are regulated by growth rate in a manner that differs from that of the other r-proteins. Genes encoding r-proteins are usually down regulated as the specific growth rate is decreased. In contrast, genes encoding the additional proteins are upregulated, as shown in Table 6 for S14 of *M. tuberculosis* grown in batch culture. A comparable result was obtained when *M. bovis* BCG was grown in continuous culture. A threefold change (from one day to three days) in the specific growth rate led to expression ratios of 0.61 for *rps* N1 and 6.0 for *rps* N2 (calculated from supplementary data of Beste *et al.*, 2007b).

5.2. Growth rate control of ribosome synthesis

Except at very low growth rates, the number of ribosomes per cell is dependent on growth rate (growth rate control of ribosome synthesis). This conclusion is based on the finding that the RNA content of cells varies with growth rate (Schaechter *et al.*, 1958; Verma *et al.*, 1999; Paul *et al.*, 2004b). As expected, the r-protein content is also dependent on growth rate. The results of microarray experiments obtained for *M. tuberculosis* and its close relative *M. bovis* BCG are presented in Fig. 2. Panel (a) provides an essential control in which the two labelled cDNA were prepared from the same RNA fraction isolated from mid-exponential cells of *M. tuberculosis* and the distribution of the expression ratios of the standard 50 r-proteins was examined. The mean value of $r_{(50)}= 1.00 \pm 0.10$ was obtained, which is a measure of the accuracy of the expression ratios. The second panel refers to cDNA prepared from mutant (*dosR* minus) and wild type *M. tuberculosis*; both mutant and wild type were found to grow at the same rate so that the ribosome contents of the two strains would be expected to be identical. In this experiment the mean value of the expression ratios of the standard set of r-proteins was found to be $r_{(50)}= 0.93 \pm 0.16$ which is similar to the control shown in panel (a). The third panel refers to *M. bovis* BCG grown in continuous culture; the specific growth rates of reference and experimental strains were 0.03 h^{-1} and 0.01 h^{-1}, respectively. In this experiment, the expression ratios were found to have a mean value of $r_{(50)}=0.71 \pm 0.01$, which indicates a lower level of r -protein mRNAs per μg cDNA in the slower growing cells and therefore a lower level of r-proteins (Cox, 2007). This conclusion is reinforced by the comparison shown in Fig. 3, which shows both the expression profiles of all 3475 ORFs investigated and the ORFs encoding the set of 50 r-proteins. The group of 50 r-protein genes form a subset that is downregulated.

6. rRNA SYNTHESIS

6.1. Organization; transcription of *rrn* operons

The organization of the two *rrn* operons of mycobacteria are shown in Fig. 4. The *rrnA* is present in all mycobacteria studied thus far. The characteristic features of *rrnA* include the numbers and locations of its promoters (Verma *et al.*, 1994; Gonzalez-y-Merchand *et al.*, 1997). This operon has a minimum of two promoters; one promoter (P1) is located within the coding region of the *murA* gene near to its 3′-end and the other (PCL1) is located near to a conserved sequence motif (CL1). Up to three additional promoters have been found, separated by 80–100 base-pairs, in some species; the *rrnA* operon of *M. smegmatis* has three promoters (P1, P2 and PCL1), *M. fortuitum* has four promoters (P1, P2, P3 and PCL1) and *M. chelonae* and *M. abscessus* each have five (P1, P2, P3, P4 and PCL1). Moreover, transcripts of *murA* continue their progress to transcribe the *rrn* operon (Gonzalez-y-Merchand *et al.*, 1999). The contributions of individual promoters to precursor-rRNA

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synthesis was found to vary according to growth rate (Gonzalez-y-Merchand *et al.*, 1998; Verma *et al.*, 1999). In brief, a single promoter tends to be dominant at high growth rates.

In contrast with *rrnA*, the *rrnB* operon is regulated by a single (P1) promoter. Transcription of *tyrS* was found to continue to progress to transcribe the *rrnB* operon (Gonzalez-y-Merchand *et al.*, 1999). The mechanism of upstream activation of the *rrnB* operon was investigated (Arnvig *et al.*, 2005). The results were found to follow the model reported for *B. subtilis* rather than the model for *E. coli*(Krásný and Gourse, 2004). *B. subtilis* rRNA promoters are much less dependent on UP elements and do not seem to use upstream-binding factors analogous to Fis. When the genome of a species encodes both *rrnA* and *rrnB*, the sequences corresponding to precursor-rRNA are very similar (see for example Sander *et al.*, 1996), but not always identical (Ninet *et al.*, 1996; Reischl *et al.*, 1998). The relatively few differences that may be present are unlikely to affect the principle features of pre-rRNA secondary structure that is processed by RNase to yield 16S rRNA, 23S rRNA and 5S rRNA. Consensus secondary structures were derived for pre-16S rRNA (Ji *et al.*, 1994a) and pre-23S rRNA and pre-5S rRNA (Ji *et al.*, 1994b; 1994c) based on comparisons of mycobacterial sequences all of which are closely related.

6.2. The stringent response

rRNA synthesis is the rate limiting step in ribosome synthesis (Paul *et al.*, 2004a) and bacteria have devised mechanisms that ensure that ribosome synthesis (and hence rRNA synthesis) is efficiently controlled to meet the cell's need for protein biosynthesis, including the need to survive under starvation conditions (the stringent response, for review see Cashel *et al.*, 1996). Much of our knowledge of the control of rRNA synthesis has come from studies of *E. coli.* Two small molecules are important regulators of rRNA synthesis in *E. coli*; nucleotide triphosphates, especially the NTP incorporated at the 5′-ends of the precursor-rRNA transcripts (Barker and Gourse, 2001) and guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and its precursor pppGpp. Two proteins encoded by *relA* and *spoT* are implicated in the synthesis of ppGpp, which is synthesised by RelA in response to uncharged tRNAs at the A-site of the ribosome (Hogg *et al.*, 2004). It is thought that ppGpp may bind directly to RNAP (for review see Paul *et al.*, 2004b). A protein DksA is essential for regulation of rRNA synthesis by NTPs and ppGpp (Paul *et al.*, 2004a). The stringent response has been studied in *M. tuberculosis* H37Rv (Primm *et al.*, 2000). Whereas *E. coli* ppGpp is synthesized through the activities of RelA and SpoT proteins, the tubercle bacillus has a single protein, Rel_{Mtb}, to achieve the same effect; that is, this single protein produces ppGpp in response to nutrient starvation. A knockout mutant (Δ rel_{Mtb}) was produced and was used to show that the Rel_{Mtb} protein is needed for the long term survival of *M. tuberculosis* under starvation conditions.

6.3. Antitermination of *rrn* transcription

The size of the pre-rRNA transcript of the *rrnA* operon of *M. tuberculosis* extending from the start site of the PCL1 promoter to the intrinsic termination site is 5,550 nucleotides. As transcription proceeds, the nascent pre-rRNA transcript will recruit r-proteins and undergo processing by nucleases. Synthesis of rRNA is the rate-limiting step in ribosome synthesis. Thus the cell is required to synthesise long pre-rRNA transcripts when ribosomes are needed. The strategy employed is to modify the RNAP complex with antitermination factors to prevent premature transcription of the *rrn* operon. A feedback mechanism is achieved if a ribosomal protein plays a role in antitermination. Particular sequence motifs first identified in phage-λ were recognized as elements in the upstream regions of *rrn* operons so that related nomenclatures (Nus (N utilizing substances) proteins and *nut* sequences) are used for both phage-λ and *rrn* operons. Transcription of the *rrn* operon requires the formation of a stable RNAP elongation complex that is able to safely ignore transcription termination

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signals. The properties of the antitermination complex identified for the N protein system of bacteriophage-λ (Mogridge *et al.*, 1995) provides the model for the composition of the antitermination complex needed to obtain full length transcripts of precursor-rRNA. The proteins identified in the phage-λ system are N, NusA, NusB, NusE (which is identical with ribosomal protein S10) and NusG. With one exception, the same proteins are needed to obtain full-length bacterial precursor-rRNA transcripts; all the Nus proteins are needed, but the cellular equivalent of the N protein has not yet been identified (Condon *et al.*, 1995).

The *nut* sequence elements are found in both the leader and ITS1 regions of rDNA. This duplication of *nut* sequences suggests that the antitermination complex is formed at the leader region, dispersed after the 16S rRNA gene is transcribed by competition for *nut* sequences by a complementary strand of RNA located downstream from the 3′-end of the 16S rRNA gene and the complex is reformed before the 23S rRNA gene is transcribed and dispersed after the transcription of the 23S rRNA gene is completed (Ji *et al.*, 1994c). The mycobacterial *nut* site within the leader region of rDNA comprises a 26 nucleotide sequence (the CL2 motif), which is present in all the mycobacteria examined so far (Gonzalez-y-Merchand *et al.*, 1997; Menendez *et al.,* 2002); the rRNA sequence is 5′-UGUUGUUUGAGAACUCAAUAGUGUGU-3′ [see Fig. 4(c)]. A similar, but not identical sequence is found in the ITS1 region.

Insights into the mechanisms of *rrn* antitermination have been advanced through physico-chemical and structural studies. A high-affinity interaction between the CL2 region of *M. tuberculosis* and its cognate NusA was reported (Arnvig *et al.,* 2004). The crystal structure of the NusA component was determined (Gopal *et al.*, 2001a). The X-ray structure of the NusA-RNA complex (Beuth *et al.*, 2005) revealed that the RNA sequence bound by NusA was 5′-AGAACUCAAUA-3′ region of the CL2 sequence [see Fig. 4(c)]. Nodwell and Greenblatt (1993) first proposed that a complex NusB and NusE (S10) interacted with the *nut* sequence element BoxA. The mycobacterial BoxA sequence occurs at the 5′-end of the CL2 motif [see Fig. 4(c)].

The *M. tuberculosis* NusB structure was determined by X -ray crystallography (Gopal *et al.*, 2000) and interaction between NusB and S10 was also demonstrated (Gopal *et al.*, 2001b). The structure of *E. coli* NusB in solution was deterined by Altieri *et al.*, (2000). NusB binds to Box A sequence directly, but its affinity for Box A is increased by interaction with NusE (S10), as shown by Das *et al.* (2008). This study strengthens the view that the ternary complex of Box A RNA/NusB/NusE (S10) interacts with the RNA polymerase complex and stabilizes it to overcome termination signals.

7. PROTEIN BIOSYNTHESIS

7.1. *In vitro* cell free systems

Translation of mRNA into protein is a complex process requiring many of the cell's resources including initiation, elongation, termination factors and the consumption of energy; the formation of each peptide bond requires the hydrolysis of four high energy phosphate bonds. Studies of mycobacterial protein synthesis were neglected until Böttger and colleagues reconstituted an *in vitro* protein synthesis system comprising *M. smegmatis* ribosomes and the complete range of factors synthesized *in vitro* from the parent genes. Fully functional hybrid ribosomes were constructed by modifying *M. smegmatis* 16S rRNA to form bacterial eukaryotic hybrids. The hybrids were formed by replacing the bacterial decoding site (helix 44) with a eukaryotic counterpart. The drug sensitivities of the hybrid ribosomes were dependent on the origin of the eukaryotic sequence (Hobbie *et al.*, 2007). The *M. smegmatis in vitro* protein synthesizing system was then used to compare the rates of reaction of individual steps in protein synthesis with the homologous reaction in *E. coli*. It

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was found that the elemental reaction rates of initiation and elongation were remarkably similar with *M. smegmatis* and *E. coli* components. The reconstituted translation system from individual purified *M. smegmatis* components is an alternative to that from *E. coli* to study mechanisms of translation and to test the action of antibiotics against Gram-positive bacteria (Bruell *et al.*, 2008).

7.2. Post-translational modification of proteins

A number of proteins possess an unusual feature termed an intein. The primary transcript contains an insertion sequence that is translated. The amino acid sequence corresponding to the intron sequence is termed an intein. 'Inteins are self-splicing elements that exist as in-frame protein fusions with two flanking sequences called exteins' (Perler *et al.*, 1994). The intein splices itself out of primary translational product leaving the two exteins which constitute the mature protein. The first mycobacterial intein recognised was found to be RecA of *M. tuberculosis*(Davis, 1991). Since then inteins were identified in three other proteins; namely, DnaB, GyrAand Pps1. Fourteen mycobacterial species are now known to have one or more intein-containing proteins (seehttp://bioinfo.weizmann.ac.il/~pietro/inteins/). The precise function of inteins is still a matter for speculation. However, the properties of inteins have been widely examined; including X-ray crystallographic and mutational studies (Van Roey *et al.*, 2007).

8. THE CELL ENVELOPE OF MYCOBACTERIA

8.1. Permeability barriers in mycobacterial cell envelopes

Nutrient uptake mechanisms obviously depend on the permeability barriers imposed by the cell envelope. Therefore, it is necessary to review the current status of the research about the cell envelope of mycobacteria. The terms are defined as follows (Beveridge, 1995; Beveridge and Kadurugamuwa, 1996; Graham *et al.*, 1991): The *cell wall* consists of the periplasm, the peptidogly can layer and, for gram-negative bacteria, the outer membrane. The *periplasmic space* represents an extracytoplasmic compartment confined between the plasma membrane and an outer structure (outer membrane versus a peptidoglycan-teichoic acid-protein network for gram-negative and gram-positive bacteria, respectively). The periplasm is composed mostly of soluble components. The *cell envelope* comprises the inner membrane and the cell wall. The components of the cell envelopes of both gram-positive and gram-negative bacteria have recently been visualized by cryo-electron microscopy (Matias *et al.*, 2003; Matias and Beveridge, 2005; Matias and Beveridge, 2006).

In microbiology textbooks, mycobacteria are classified as gram-positive bacteria. However, it is well documented that mycobacteria, unlike other gram-positive bacteria, have evolved a very complex cell wall, comprising a peptidoglycan-arabinogalactan polymer with covalently bound mycolic acids of considerable size (up to 90 carbon atoms), a large variety of extractable lipids (Barry *et al.*, 1998; Daffé and Draper, 1998) and pore-forming proteins (Niederweis, 2003). Most of the mycobacterial lipids are constituents of the cell envelope, which provides an extraordinarily efficient permeability barrier to noxious compounds, rendering mycobacteria intrinsically resistant to many drugs (Brennan and Nikaido, 1995). Due to the paramount medical importance of *M. tuberculosis*, the ultrastructure of mycobacterial cell envelopes has been intensively studied for decades by electron microscopy. To account for the extraordinary efficiency of the mycobacterial cell wall as a permeability barrier, Minnikin originally proposed a model in which the mycolic acids are covalently bound to the peptidoglycan–arabinogalactan co-polymer and form the inner leaflet of an asymmetrical bilayer (Minnikin, 1982). Other lipids extractable by organic solvents were thought to form the outer leaflet of this outer bilayer. X-ray diffraction studies of mycobacterial cell walls showed that the mycolic acids are oriented parallel to each other

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and perpendicular to the plane of the cell envelope (Nikaido*et al*, 1993). This provided experimental support for some fundamental aspects of the Minnikin model. Mutants and treatments affecting mycolic acid biosynthesis and the production of extractable lipids showed an increase of cell wall permeability and a drastic decrease of virulence, underlining the importance of the integrity of the cell wall for intracellular survival of *M. tuberculosis* (Barry *et al*., 1998). These indirect structural, biochemical and genetic data are consistent with the existence of an outer lipid bilayer as proposed by Minnikin (1982). However, this model faced criticism mainly because electron microscopy of mycobacteria, in particular thin sections thereof, never showed evidence for an additional, outer lipid bilayer (Daffé and Draper, 1998; Draper, 1998). Recently, we have visualized the envelope of intact *M. smegmatis* and *M. bovis* BCG cells by cryo-electron tomography and have provided, for the first time, direct and final evidence for the existence of this structure (Hoffmann *et al*., 2007; Hoffmann *et al*., 2008). An important result was that cell envelopes of *M. smegmatis* and *M. bovis* BCG a re very similar both in appearance and in dimensions. Thus, it makes sense to name this unusual structure “mycobacterial outer membrane” (Hoffmann *et al*., 2008). Cryo-electron tomography and cryo-electron microscopy of ultrathin sections clearly showed that the mycobacterial outer membrane is a bilayer structure. A mycolic acid-deficient mutant of *Corynebacterium glutamicum*, a close relative of mycobacteria, did not produce an outer membrane demonstrating that mycolic acids are an essential component of the outer membrane in these bacteria (Hoffmann *et al*., 2008). The results of this study were subsequently confirmed (Zuber *et al*., 2008) and are summarized in Fig. 5. Biologically very important consequences of these findings are that mycobacteria must have proteins that functionalize the periplasm and the outer membrane.

8.2. Transport across mycobacterial outer membranes

The utilization of many solutes such as carbohydrates, alcohols, carboxy acids, fatty acids and amino acids by mycobacteria was originally examined as early as 1951 (Edson, 1951). It is therefore striking that the identity of many transporters for essential nutrients of *M. tuberculosis* and other mycobacteria are still unknown despite considerable progress in the development of genetic methods for mycobacteria (Kana and Mizrahi, 2004; Machowski *et al*., 2005). Recently, significant progress has been made to understand the uptake of some nutrients both in *M. smegmatis* and *M. tuberculosis*. For example, proteins were identified and characterized that are involved in uptake of several nutrients such as phosphate (Gebhard *et al*., 2006; Peirs*et al*., 2005; Vyas *et al*., 2003; Webb, 2003; Wolschendorf*et al*., 2007), sulphate (Wooff *et al*., 2002) and some amino acids (Seth and Connell, 2000; Talaue *et al*., 2006). The current knowledge about nutrient uptake across both inner and outer membranes in mycobacteria was recently summarized (Niederweis, 2008a; Niederweis, 2008b) and is updated here. A putative link between outer membrane transport and growth rate of mycobacteria has recently been discussed (Cox and Cook, 2007).

8.3. Porin-mediated diffusion of hydrophilic solutes

Porins are defined as non-specific protein channels in bacterial outer membranes which enable the influx of hydrophilic solutes (Nikaido, 2003). Channel-forming proteins that are functionally similar toporins of gram-negative bacteria have been observed in many mycobacteria (Niederweis, 2003) and other closely related genera such as corynebacteria. MspA was discovered as the first porin of *M. smegmatis* (Niederweis *et al*., 1999). Deletion of *mspA* reduced the outer membrane permeability of *M. smegmatis* towards cephaloridine and glucose nine -and four -fold, respectively (Stahl *et al*., 2001). These results show that MspA is the major general diffusion pathway for hydrophilic solutes in *M. smegmatis*. Consecutive deletions of the two porin genes *mspA* and *mspC* reduced the number of pores by 15 -fold compared to wild-type *M. smegmatis*. The loss of porins lowered the permeability for glucose by 75-fold and, concomitantly, the growth rate of *M. smegmatis* on

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plates and in liquid medium dropped drastically (Stephan *et al.*, 2005). This showed for the first time that the porin-mediated influx of hydrophilic nutrients limited the growth rate of porin mutants. However, it is unknown which and how many nutrients are in low supply in the *M. smegmatis* porin mutants. Since MspA could not be express ed in *M. smegmatis* above wild-type levels (Stephan *et al.*, 2005), it is also not clear whether the influx of nutrients really limits the growth rate of wild-type mycobacteria as suggested earlier (Jarlier and Nikaido, 1990). The fact that the lack of the MspA and MspC porins also caused a reduced uptake of phosphates and slower growth on low-phosphate plates (Wolschendorf *et al.*, 2007) indeed suggested that the slow uptake of essential hydrophilic nutrients other than the carbon source may also contribute to the slow growth of *M. smegmatis* porin mutants.

The crystal structure of MspA represents the first crystal structure of any mycobacterial outer membrane protein and revealed an octameric goblet-like conformation with a single central channel of 10 nm in length (Faller *et al.*, 2004). This structure is different from that of the trimeric porins of Gram-negative bacteria, which have one pore per monomer and are approximately 5 nm long (Koebnik *et al.*, 2000). Its structural features define MspA as the founding member of a new class of outer membrane proteins. The crystal structure also revealed that the constriction zone of MspA consists of 16 aspartates (D90/D91). Thus, the zone of MspA with the smallest diameter is highly negatively charged (Faller *et al.*, 2004). This most likely explains the previously observed preference of MspA for cations (Kartmann *et al.*, 1999). The MspA pore provides an example of how outer membrane transport proteins can contribute to the selectivity of mycobacteria towards particular nutrients.

The existence of channel-forming proteins in *M. tuberculosis* and in *M. bovis* BCG has been demonstrated (Kartmann *et al.*, 1999; Lichtinger *et al.*, 1999; Senaratne *et al.*, 1998). Uptake of serine, but not of glycine, was reduced in the *ompATb* mutant compared to wild-type *M. tuberculosis.* This was interpreted as proof that OmpATb is a porin consistent with its apparent channel-forming activity *in vitro* (Raynaud *et al.*, 2002b). However, the overall permeability of the outer membrane of *M. tuberculosis* was reduced at pH 5.5 compared to pH 7.2, although the levels of OmpATb in the outer membrane were strongly increased (Raynaud *et al.*, 2002b). Considering these contradicting results, it is doubtful that OmpATb has significant porin function in *M. tuberculosis* (Niederweis, 2003). The observation that a central domain of approximately 150 amino acids is sufficient for the channel activity of OmpATb *in vitro* does not contribute to the understanding of its biological functions (Molle *et al.*, 2006).

Rv1698 was annotated as a protein of unknown function and was predicted as an outer membrane protein of *M. tuberculosis* (Lee *et al.*, 2008). Lipid bilayer experiments of purified protein and uptake experiments in a porin mutant of *M. smegmatis* demonstrated that Rv1698 is a channel-forming membrane protein (Siroy *et al.*, 2008). Its surface accessibility was shown by protease experiments in whole cells (Song *et al.*, 2008). A homolog of Rv1698 exists in all mycolic acid containing bacteria. Hence, Rv1698 represents the first member of a new class of channel proteins specific for mycolic acid-containing outer membranes.

8.4. Direct diffusion of hydrophobic solutes through the cell membranes

Hydrophobic molecules, in particular nonelectrolytes, can easily diffuse through phospholipid bilayers. However, the lipopolysaccharide-containing outer membrane of Gram-negative bacteria constitutes a considerable permeability barrier which does not allow the penetration of even extremely hydrophobic β-lactam antibiotics (Nikaido *et al.*, 1983). The lipids in mycobacterial cell walls are likely to be organized in a very unusual, asymmetric bilayer (Nikaido *et al.*, 1993). Differential scanning calorimetry showed that the

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lipids in mycobacterial cell walls have very high phase transition temperatures in the range of 60 – 70 °C. This is suggestive of a lipid domain of extremely low fluidity (Liu *et al.*, 1995). Isolated cell walls of corynebacteria, which contain much shorter corynemycolic acids, displayed a much lower temperature transition, suggesting that the fluidity of this lipid bilayer is mainly determined by the mycolic acids. Since direct diffusion of a hydrophobic molecule through a lipid membrane requires that it is dissolved in the lipid phase, the permeability of a particular membrane is directly correlated with its fluidity. This has been demonstrated directly by Nikaido and co-workers (Andersson *et al.*, 1996). It is concluded that the mycobacterial outer membrane presents a strong permeability barrier for hydrophobic molecules. On the other hand, there is emerging evidence that fatty acids rather than carbohydrates might be the dominant carbon source of *M. tuberculosis* after the onset of the immune response. This includes the requirement for isocitrate lyase for growth and persistence of *M. tuberculosis* in macrophages and in mice (McKinney *et al.*, 2000), and the induction of expression of genes encoding enzymes involved in the β-oxidation of fatty acids in macrophages (Schnappinger *et al.*, 2003) and mice (Dubnau *et al.*, 2005; Timm*et al.*, 2003). However, it is unknown how fatty acids are transported across the outer membrane of mycobacteria.

9. TRANSPORT ACROSS THE INNER MEMBRANE

9.1. Transporters of carbohydrates

It has been widely documented that *M. smegmatis* can grow on many carbon sources such as polyols, pentoses and hexoses (Edson, 1951; Franke and Schillinger, 1944; Izumori et al., 1976). A recent comprehensive analysis of carbohydrate uptake systems revealed that the soil bacterium *M. smegmatis* has 28 putative carbohydrate transporters (Titgemeyer *et al.*, 2007). The majority of sugar transport systems (19/28) in *M. smegmatis* belongs to the ATP-binding cassette (ABC) transporter family (Fig. 6A). *M. smegmatis* further possesses one putative glycerol facilitator of the major intrinsic protein (MIP) family, four sugar permeases of the major facilitator super family (MFS) of which one was assigned as a glucose transporter, and one galactose permease of the sodium solute super family (SSS). Thus, inner membrane transport systems for polyols, pentoses and hexoses are predicted to exist in *M. smegmatis* (Fig. 6A). This bioinformatic prediction was proven recently by the discovery of glucose permease GlcP (Msmeg_4182) from *M. smegmatis* mc^2155. Upon overexpression of this protein in a glucose-negative mutant of *E. coli* it was shown that the strain was capable of glucose fermentation in addition to increased uptake of glucose (Pimentel-Schmitt *et al.*, 2008). Therefore, GlcP is a first known example of a high-affinity sugar permease ($K_m$of 19 μM) found in mycobacteria.

M. smegmatis does not grow on lactose, maltose and sucrose as a sole carbon source (Titgemeyer *et al.*, 2007). Franke and Schillinger obtained the same result for lactose and maltose, but observed respiration of *M. smegmatis* in the presence of sucrose (Franke and Schillinger, 1944). *M. smegmatis* has at least three inner membrane transport systems with significant similarities to other bacterial disaccharide transporters (Niederweis, 2008b). However, the substrate specificities of the transporters encoded by *msmeg0501-0508* and *msmeg0509-0517* are not known. Growth of bacteria on disaccharides as sole carbon sources requires enzymes, which cleave the disaccharide and release the monosaccharides for further utilization. The absence of proteins similar to known bacterial β-D-galactosidases (LacZ of *E. coli*, BgaB of *B. circulans*, MbgA of *B. megaterium*, LacA *S. coelicolor*) provides a molecular explanation for the inability of *M.s megmatis* to utilize lactose as a sole carbon source. By contrast, *M. smegmatis* has six homologs (MSMEG3191, 3577, 4901, 4902, 4685, 6477) of MalL of *B. subtilis*, which hydrolyzes maltose, longer maltodextrines up to maltohexose, isomaltose and sucrose (Schönert *et al.*, 1999), and of the cytoplasmic trehalase TreC of *E.coli*, which cleaves trehalose-6-phosphate (Rimmele and

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Boos, 1994). It is conceivable that these enzymes are used in trehalose metabolism considering the unusual importance of trehalose in mycobacteria (Murphy *et al.*, 2005; Woodruff *et al.*, 2004) and the observation that trehalose was the only disaccharide which was used by *M. smegmatis* as a sole carbon source. However, it cannot be excluded that some of the enzymes with similarities to TreC and MalF have roles in pathways distinct from trehalose metabolism.

Bioinformatic analysis of the genome of *M. tuberculosis* H37Rv revealed four ABC-type transporters and one permease of the MFS class for carbohydrates (Titgemeyer *et al.*, 2007) (Fig. 6B). These ABC transporters have been described earlier in a global analysis of the *M. tuberculosis* genome (Braibant *et al.*, 2000; Content *et al.*, 2005). It is obvious that *M. tuberculosis* is poorly equipped with carbohydrate transport systems in comparison to *M. smegmatis*. Two of the operons, the *lpgY sugABC* and the *uspABC* operons, are highly conserved between the two species. The proteins of the ABC^{Sug} and of the ABC ^{Usp} systems share between 62% and 80% similar amino acids, compared to only 25 to 30% similar amino acids for the ABC^{Ugp} and the Rv2038c/Rv2039c/R v2040c/Rv2041c systems. The similarities of all four ABC systems to known transporters outside the genus *Mycobacterium* is so low (< 25%) that substrates of these transporters cannot be predicted (Titgemeyer *et al.*, 2007).

The ABC^{Sug} sugar transport system was predicted to be essential for virulence of *M. tuberculosis* in mice based on transposon site hybridization (TraSH) experiments (Sassetti *et al.*, 2003). Previously, it was suggested that this permease may transport maltose or maltodextrins (Borich *et al.*, 2000; Braibant *et al.*, 2000). However, both the similarities of ABC^{Sug} and of the corresponding substrate binding protein LpgY to the maltose transporters and periplasmic maltose binding proteins MalE of *E. coli* and *S. coelicolor* are very low (< 25%). Thus, it is questionable whether maltose is the substrate of ABC^{Sug}. This conclusion is supported by the fact that neither *M. smegmatis*, which has a highly similar ABC^{Sug} system, nor *M. tuberculosis* (Edson, 1951) grow on maltose as a sole carbon source. It has to be noted that similar uncertainties exist about the substrate specificities of the four other carbohydrate uptake system of *M. tuberculosis* including the ABC^{Usp} transporter which was proposed to transport sn-glycerol-3-phosphate based on low protein similarities (Braibant *et al.*, 2000; Content *et al.*, 2005). The SugI transporter of the MFS class shows distant sequence similarity to the glucose permease GlcP (28%) of *S. coelicolor* and to the galacto se (GalP, 24%) and arabinose (AraE, 24%) transporters of *E. coli*. Thus, the SugI system may transport a monosaccharide.

Glycerol is used as the standard carbon source to grow *M. tuberculosis*, however no uptake system is known or apparent by sequence similarity (Titgemeyer *et al.*, 2007). Since *M. tuberculosis* grows with a generation time of 24 hours and it has been shown that glycerol can directly diffuse through lipid membranes both *in vitro* (Paula *et al.*, 1996) and *in vivo* (Eze and McElhaney, 1981), it is conceivable that the rate of glycerol intake by passive diffusion may be sufficient for growth. Incoming glycerol would then be converted by glycerol kinase (GlpK) to glycerol-3-phosphate to enter the route of central carbon metabolism (Fig. 6B). *M. tuberculosis* has one putative glycerol kinase that shows a high similarity to the two glycerol kinases of *M. smegmatis* (MSMEG_6759, 77% and MSMEG_6756, 57%) and to the two glycerol kinases from *S. coelicolor* SCO0509 (75%) and SCO1660 (59%).

9.2. Comparison of inner membrane sugar transporters of *M. smegmatis* and *M. tuberculosis*

The analysis of the carbohydrate uptake proteins in the genomes of *M. smegmatis* and *M. tuberculosis* confirms the very early phenotypical observations that the saprophytic

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mycobacteria have a much broader spectrum of substrates, which they can use as sole carbon and energy sources (Edson, 1951). It is striking that the genome of *M. tuberculosis* has only five permeases for carbohydrate uptake compared to 28 of *M. smegmatis*. This suggests that the phagosome does not provide an environment rich in diverse sugars. The tantalizing conclusion is that an experimental analysis of the substrate specificity of the inner membrane carbohydrate transporters of *M. tuberculosis* may reveal the carbon sources available in the phagosome of human macrophages and/or in other cellular hideouts of *M. tuberculosis* inside the human body.

9.3. Transporters of lipids

Several lines of evidence strongly suggest that *M. tuberculosis* switches from a carbohydrate to a fat diet after the onset of the adaptive immune response. (i) Biochemical studies suggest that in chronically infected lung tissues, fatty acids might be a major source of carbon and energy for *M. tuberculosis* (Wheeler *et al.*, 1990). (ii) During the first 10 days of infection of mice, *M. tuberculosis* requires the sugar transporter SugAB for survival (Sassetti *et al.*, 2003). Thereafter, enzymes such as isocitratelyase and malatesynthases are essential for virulence (McKinney *et al.*, 2000). This indicates that lipids are the major carbon and energy source of *M. tuberculosis* because the glyoxylate shunt is required for running the citric acid cycle on acetate which is produced by degradation of lipids through β-oxidation. (iii) *M. tuberculosis* possesses four genes encoding putative phospholipases C, *plcA*, *plcB*, *plcC* and *plcD*. These genes are required for virulence of *M. tuberculosis* in mice (Raynaud*et al.*, 2002a). The fact that the phospholipases C are attached to the cell wall by lipid anchors argues for a role of these enzymes in the controlled release of fatty acids likely from phospholipids of the phagosomal membrane. However, while several proteins have been identified in *M. tuberculosis* involved in the transport of lipids from the cytoplasm to the outer membrane (Jackson *et al.*, 2007), the proteins involved in the transport of lipids across the outer membrane are yet unknown. By contrast, the mechanisms that govern the uptake of exogenous fatty acids are well established in *E. coli* (Dirusso and Black, 2004). When the cell encounters long chain fatty acids in the environment, these ligands bind to outer membrane protein FadL and via a ligand-induced conformational shift within the protein are transported into the periplasmic space. The more acidified environment of the periplasmic space promotes the formation of uncharged fatty acid molecules, which partition into and flip across the inner membrane. Within the cytosol, the acyl-CoA synthase FadD partitions into the inner membrane, where it functions in the vectorial esterification of the long chain fatty acids (Dirusso and Black, 2004). Consistent with the importance of lipid uptake, *M. tuberculosis* possesses numerous homologs of FadD proteins (Trivedi *et al.*, 2004). Moreover, an 82-gene cluster (Rv3492c-Rv3574) responsible for catabolism of cholesterol including at least one set of genes that encode all enzymes necessary to perform the full cycle of β-oxidation and a multicomponent cholesterol uptake system consisting of *mce4ABCDEF* and an ABC-transporter *supAB* has been found in *M. tuberculosis* (Van der Geize *et al.*, 2007). It was shown that Mce4 forms the major cholesterol import system of *M. tuberculosis*, which can be used by the cell for both carbon and energy production as a primary carbon source (Pandey and Sassetti, 2008). Furthermore, mutation in *mce4* operon leads to attenuation during chronic phage of infection in mouse spleen and lungs and in activated, but not resting macrophages, suggesting that uptake of lipids function as a major carbon source at the later stage of *M. tuberculosis* infection (Joshi *et al.*, 2006; Pandey and Sassetti, 2008; Sassetti *et al.*, 2003).

9.4. Transporters of phosphorus-containing solutes

Phosphorus is indispensable for energy supply, for the biosynthesis of nucleic acids and phospholipids, and many other cellular processes. While inorganic phosphate is the preferred source of phosphorous, many bacteria can also take up organic phosphates and

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release phosphate by the action of periplasmic phosphatases such as PhoA. Gram-negative bacteria employ sophisticated transport mechanisms to acquire phosphorus containing nutrients from the environment. *E.coli* uses four phosphate transport systems Pst, Pit, GlpT and UhpT to translocate inorganic phosphate across the inner membrane (van Veen, 1997). Part of the Pst system is the periplasmic protein PstS, which binds and transfers phosphate to the transmembrane components PstA and PstC. PstB hydrolyzes ATP and delivers energy for phosphate translocation across the inner membrane by PstA/PstC. Pst systems from gram-negative bacteria bind and transport phosphate with binding constants and apparent transport K_m values in the submicromolar range. *M. tuberculosis* contains several copies of the genes encoding the Pst system (Braibant *et al.*, 1996). Two Pst components, PstS1 and PstS2, have been shown to be virulence factors in *M. tuberculosis* (Peirs *et al.*, 2005; Sassetti *et al.*, 2003). Further, *M. tuberculosis* contains two genes, *pitA* and *pitB,* which encode putative constitutive inorganic phosphate transporters (Content *et al.*, 2005a). The physiological role of the Pit transporters is unclear.

The single *pstSCAB* operon of the fast-growing *M. smegmatis* encodes a high-affinity Pst system with an apparent $K_m$value of 40 μM phosphate. A second high-affinity phosphate uptake system of *M. smegmatis* is encoded by the *phnDCE* operon (Gebhard *et al.*, 2006). However, even a *phnD pstS* double mutant did not show a reduced phosphate uptake suggesting the presence of a third high-affinity phosphate uptake system of *M. smegmatis* (Gebhard *et al.*, 2006). Considering the presence of three high-affinity phosphate uptake systems, which are inducible at low phosphate concentration in *M. smegmatis*, it is unclear why a *pstB* mutant showed a reduced phosphate transport (Bhatt *et al.*, 2000). Taken together, these results underline the importance of phosphate uptake for mycobacteria. The transcriptional profiles of *M. tuberculosis* and *Salmonellaenterica* in infected macrophages revealed that the proteins involved in inorganic phosphate transport are up-regulated (Eriksson *et al.*, 2003; Schnappinger *et al.*, 2003) indicating that phosphate levels inside phagosomes of macrophages are indeed limited. Consistent with this conclusion, genes encoding efficient phosphate transport systems were found to be essential for the survival of *M. tuberculosis* in macrophages and mice (Rengarajan *et al.*, 2005; Sassetti *et al.*, 2003). However, it is unknown how inorganic or organic phosphates cross the outer membrane of *M. tuberculosis.* Since direct diffusion of phosphates through model lipid membranes is extremely slow (permeability coefficient of the mono anion: 5×10^{-12} cm/s (Chakrabarti and Deamer, 1992)) it appears likely that slowly growing mycobacteria also use outer membrane pore proteins for uptake of phosphate. Indeed, the existence of a porin with anion specificity has been demonstrated (Lichtinger *et al.*, 1999). This porin still awaits discovery.

9.5. Transporters of sulfur-containing solutes

Sulfur is essential in cells for biological activities such as translation initiation and maintenance of the redox potential. Transposon insertions in the *cysA* and *subI* genes of *M. bovis* BCG yielded methionine auxotrophs. These mutants were resistant to chromate and did not take up sulfate. These results identified the products of the genes *cysTWA* and *subI* as components of a sulphate permease and indicated that this transporter is the sole sulphate transporter of *M. bovis* BCG (McAdam *et al.*, 1995; Wooff *et al.*, 2002). The sensitivity of sulphate uptake to azide and 1,3-dicyclohexylcarbodiimide are characteristic of ABC transporters. Survival of the *cysA* and *subI* mutants in mice was not different from wild-type *M.bovis* BCG. This indicated that, in the host, methionine may be a more important sulfur source than sulphate for growth of the *M. tuberculosis* complex. This may also explain how *M. leprae* remains a pathogen, despite being a natural methionine auxotroph because of its loss of *cysTWA* for sulphate transport (Wood, 1995). An alternative explanation may be that other putative sulphate transporters such as the predicted sulphate permease (SulP) of *M. tuberculosis* are induced *in vivo* and compensate for the loss of the CysTWA transporter

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(Content *et al.*, 2005a). To this end, it was shown that overexpression of SulP protein Rv1739c in *E. coli* leads to increased uptake of sulfate by intact cells with its maximum at pH 6.0 (Zolotarev *et al.*, 2008). However, to prove the function of this protein as a sulfate transporter of *M. tuberculosis*, construction of knock-out mutant and sulfate uptake experiments are required.

9.6. Transporters of nitrogen-containing solutes

Nitrogen is an essential component of nearly all complex macromolecules in a bacterial cell, such as proteins, nucleic acids and cell wall components. Ammonium is the preferred nitrogen source of many bacteria. In enteric bacteria, diffusion of uncharged ammonia (NH_3) through the cytoplasmic membrane into the cell is sufficient to support growth in the presence of high amounts of ammonium (NH_4+) in the growth medium. Only when diffusion across the cell envelope becomes limiting for growth is the ammonium transporter AmtB synthesized. As in enteric bacteria, mycobacteria possess homologues of AmtB (Nolden *et al.*, 2001). However, no biochemical data are available for ammonium uptake by mycobacteria and the role of AmtB in this process. Two ammonium transporters, AmtB (Msmeg_2425) and Amt1 (Msmeg_6259), have been proposed to take up ammonium in *M. smegmatis* based on their transcriptional regulation by GlnR, a key regulator of nitrogen control in mycobacteria, and RT-PCR analysis under nitrogen limited conditions (Amon *et al.*, 2008). However, direct evidence on involvement of *amtB* and *amt1* into transport of ammonium in *M. smegmatis* is missing.

Nitric oxide (NO) is generated in large amounts within the macrophages and restricts the growth of *M. tuberculosis.* Nitrate can be produced by oxidation of nitric oxide and is an alternative source of nitrogen for bacteria within the human host. Early work in *E. coli* had suggested that *narK* was involved only in nitrite export (Rowe *et al.*, 1994), and so the homologous *narK2* in *M. tuberculosis* was annotated as a “nitrite extrusion protein”. More recent work with an *E. coli narK narU* double mutant indicated that the two proteins could transport nitrate into and nitrite out of the cell (Clegg *et al.*, 2002; Jia and Cole, 2005). In *M. tuberculosis*, four genes, *narK1* through *narK3* and *narU* are homologous to *narK* and *narU*. Since *M. tuberculosis* is unable to reduce nitrite, which could accumulate to toxic levels, it must be exported out of the cell. The *M. tuberculosis narK2* was shown to complement this *E. coli* double mutant, supporting a role for *narK2* in nitrate reduction by coding for a transporter of nitrate into and nitrite out of the cell (Sohaskey and Wayne, 2003). Nitrate reduction by *M. tuberculosis* is regulated by control of nitrate transport into the cell by NarK2. It is proposed that NarK2 senses the redox state of the cell, possibly by monitoring the flow of electrons to cytochrome oxidase, and adjusts its activity so that nitrate is transported under reducing, but not under oxidizing, conditions (Sohaskey, 2005). Inhibition of nitrate transport by oxygen has been documented in other bacteria (Moir and Wood, 2001). It is intriguing that *M. tuberculosis*, classified as an obligate aerobe, should have such intricate control of an anaerobic enzyme system. Transcription of *narK2* is controlled by DosR/DevR, which responds to oxygen (O_2), nitric oxide (NO), and carbon monoxide (CO) (Kumar *et al.*, 2008; Ohno *et al.*, 2003; Voskuil *et al.*, 2003). Both the transcription of the *narK2* gene and the activity of NarK2 are controlled by similar signals (Sohaskey, 2005).

9.7. Transporters of amino acids

Many microorganisms use amino acids as a source of energy and/or nitrogen, and also for biosynthetic purposes. It was shown early in seminal papers by Yabu that D-amino acids are taken up rapidly by mycobacteria while the L-forms are transported at a much lower rate (Yabu, 1967; Yabu, 1970; Yabu, 1971). These results can be attributed to the specificity of the inner membrane transporters for the natural form of amino acids. It was also found early

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that *M. tuberculosis* cannot utilize amino acids to support metabolism in contrast to saprophytic mycobacteria (Edson, 1951).

Nevertheless, some amino acids are taken up by *M. tuberculosis* and *M. bovis* BCG (Seth and Connell, 2000). In particular, uptake of arginine was examined because arginine plays also an important role in the cellular immune response as the substrate of the inducible nitric oxide synthase (iNOS) which generates nitric oxide to kill bacterial and parasitic pathogens in macrophages (Chan *et al.*, 2001; Nathan and Shiloh, 2000). Thus, competition between the pathogen and macrophages for arginine has been suggested to contribute to the outcome of infection (Mills, 2001). Not surprisingly, *M. tuberculosis* has several genes encoding putative L-arginine uptake transporters: Rv0522, Rv1979c, Rv1999c, Rv2320c and Rv3253c (Cole *et al.*, 1998). Transport of L-arginine, but not of L-lysine and L-ornithine, was reduced by 70% in a mutant of *M. bovis* BCG lacking the gene homologous to *rv0522*. This identified Rv0522 (GabP) as an arginine transporter of *M. tuberculosis* (Seth and Connell, 2000). The remaining 30% of L-arginine transport activity and the uptake of other cationic amino acids by the mutant are likely mediated by other amino acid permeases.

Some amino acids such as proline and glycine betaine are important in regulating the osmotic pressure in bacterial cells (Wood *et al.*, 2001). Recently, it was demonstrated that the ABC transporter ProXVWZ imports glycine betaine and protects *M. tuberculosis* against osmotic stress and thereby provides a growth adavantage both *in vitro* and in macrophages (Price *et al.*, 2008).

9.8. Transporters of inorganic cations

Metal ions such as Fe^{2+}/Fe^{3+}, Cu^{+}/Cu^{2+} and Zn^{2+} play structural and catalytic roles in metalloenzymes. Genome analysis of *M. tuberculosis* revealed 28 genes encoding a broad repertoire of putative metal ion transporters. They comprise eight families of secondary active transporters and three families of primary active transporters, including twelve 'P' type ATPases, and represent approximately one quarter of all transporters in this organism. Potential metal ion specificities include K^{+}, Na^{+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, $Fe^{2+/3+}$, Hg^{2+}, AsO_2^{-} and AsO_4^{3-}. Seventeen of these transporters are also encoded as complete open reading frames in *Mycobacterium leprae*, suggesting a role in intracellular survival. The properties of these transporters, including the NRAMP orthologue MntH which transports manganese ions in other bacteria, has been reviewed recently (Agranoff and Krishna, 2004; Content *et al.*, 2005). Here we summarize only the new findings for the uptake of iron by *M. tuberculosis.*

Throughout the living world, iron is contained in the active centers of most redox enzymes. Because iron occurs in the insoluble Fe^{3+}form under oxic conditions (10^{-9}M Fe^{3+}in soil and water) (Ratledge and Dover, 2000), proteins and siderophores with high binding affinity are required to make Fe^{3+}biologically available. *M. tuberculosis* produces salicylate-containing siderophores named mycobactins. The more polar form (carboxymycobactin) is released into the medium, whereas the less polar form (mycobactin) remains cell-associated (Ratledge and Dover, 2000). Upon binding by siderophores, $Fe^{3+}$is transported into the bacterium and released from the siderophore, possibly by reduction. In most bacteria, Fe^{3+}-siderophore complexes bind to specific receptor proteins on the cell surface and are actively transported into the cytoplasm by specialized proteins that belong to the family of ATP-binding cassette (ABC) transporters (Braun and Killmann, 1999). It is suggested that three proteins, IrtA, IrtB and Rv2895c, are mainly involved in iron homeostasis in *M. tuberculosis*(Farhana *et al.*, 2008). The ABC transporter IrtAB is required by *M. tuberculosis* to replicate in iron-deficient medium and to use carboxymycobactin as an iron source, indicating that IrtAB is involved in the transport of this compound (Rodriguez, 2006). Further evidence suggested that IrtA is a carboxymycobactin exporter based on *in vitro*

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reconstitution experiments, while the two-component IrtB-Rv2895c system functions as importer of Fe^{3+}-carboxymycobactin. A knockout mutant of *msmeg_6554*, the *irtA* homologue in *M. smegmatis*, displayed an impaired siderophore export that is restored upon complementation with *M. tuberculosis irtA* (Farhana *et al.*, 2008). Moreover, deletion of the *irtAB* genes in *M. tuberculosis* reduced its ability to survive in macrophages and in the lungs of mice. However, the lack of *irtAB* does not completely eliminate replication of *M. tuberculosis* in iron-deficient conditions, which indicates that other transporters can partially compensate for the lack of IrtAB (Rodriguez and Smith, 2006). Given the importance of iron, this is not surprising because pathogenic bacteria often have multiple pathways for iron acquisition. The genome of *M. tuberculosis* does not reveal other obvious siderophore transporters (Rodriguez, 2006). However, there are numerous ABC transporters, the substrates of which are unknown; therefore, one or more of these could contribute to iron uptake.

10.0 SENSORY PERCEPTION

Mycobacteria possess an extraordinary ability to adapt to and survive under adverse conditions, including nutrient deprivation, hypoxia, various exogenous stress conditions and, in the case of the pathogenic species, the intraphagosomal environment. A key requirement for this resilience is sensory perception of the surrounding conditions and the composition of the extracellular milieu. The genome of *M. tuberculosis* contains an array of signal transduction systems, including 11 complete two-component regulatory systems, 14 eukaryotic type serine/threonine protein kinases (STPKs) and phosphatases, and ten extracytoplasmic function (ECF) sigma factors (Cole *et al.*, 1998), which have been subject of several recent reviews (Manganelli *et al.*, 2004; Tucker *et al.*, 2007; Tyagi and Sharma, 2004; Wehenkel *et al.*, 2008), as well as an unusually high number of nucleotide cyclases (McCue *et al.*, 2000). To date, only a few of these systems have been assigned a signal they respond to, reflecting the fact that very little is known about stimulus perception in these bacteria. The numbers of regulatory systems vary between species of mycobacteria, e.g. with *M. smegmatis* containing only six of the 11 STPKs of *M. tuberculosis*, but up to 23 ECF sigma factors (Tyagi and Saini, 2004; Tyagi and Sharma, 2004; Waagmeester *et al.*, 2005) and *M. leprae* containing only four STPKs and thr ee ECF sigma factors (Cole *et al.*, 2001; Tyagi and Saini, 2004; Tyagi and Sharma, 2004). These differences do not always correlate with differences in genome size, but may rather reflect specific adaptations to lifestyle, i.e. pathogenic or environmental, and the various conditions encountered in the respective environments. The mechanisms by which mycobacteria sense different aspects of their environment are discussed below.

10.1 Sensing nutrient availability

10.1.1 Carbon source—Mycobacteria can use a wide variety of carbon sources, such as carbohydrates, fatty acids and lipids or the carbon backbones of amino acids (Wheeler, 2005). Fatty acids are thought to constitute the main source of carbon for mycobacteria within the host, and the switch from carbohydrate utilization to fatty acid utilization is crucial for the successful establishment of persistent infection (Honerzu Bentrup and Russell, 2001; McKinney *et al.*, 2000; Schnappinger *et al.*, 2003). Despite the importance of this adaptation, little is known about how mycobacteria sense the availability and adjust their metabolism to the utilization of the available carbon source. Microarray analysis showed several genes involved in fatty acid metabolism, e.g. *icl* and *fadB2*, to be up-regulated after exposure of *M. tuberculosis* to acidic pH (Fisher *et al.*, 2002). The authors proposed that a drop in pH, which the bacteria will encounter upon entry into the phagosome, may constitute an indirect signal that the cell has now entered the host and will need to switch its metabolism to the utilization of fatty acids (Fisher *et al.*, 2002). However,

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Hampshire and colleagues found genes for fatty acid metabolism upregulated during late-stationary phase of an *M. tuberculosis* culture grown on glucose and glycerol (Hampshire *et al.*, 2004). These experiments were done with careful control of temperature, oxygen-tension and pH, and therefore the signal that led to induction of fatty acid metabolism genes was most likely directly related to the depletion of carbohydrates. In another study, six of the 18 genes for fatty acid degradation, which were found to be induced in *M. tuberculosis* grown within murine bone-marrow macrophages, were also found to be induced *in vitro* during growth on palmitic acid (Schnappinger *et al.*, 2003), again suggesting a direct perception of the carbon-source available for growth. Furthermore, utilization of fatty acids as carbon sources is not restricted to pathogenic species of mycobacteria, and it is therefore unlikely that an indirect signal associated with entry into the phagosome provides the sole stimulus for the appropriate change in gene expression.

A strain of *M. tuberculosis* in which expression of the membrane-bound STPK PknF had been knocked down using antisense RNA displayed an increase in glucose uptake, but no difference in uptake of other carbon sources such as leucine or oleic acid (Deol *et al.*, 2005). The authors therefore proposed an involvement of PknF in the repression of sugar transport. However, PknF was not found to be differentially expressed in a nutrient starvation model of *M. tuberculosis*, where key enzymes of glycolysis and the TCA cycle were down-regulated (Betts *et al.*, 2002), suggesting that alternative mechanisms for the regulation of carbon source utilization must exist. An adenylyl cyclase of *M. tuberculosis*, Rv2212, has been shown to respond to pH, and this response is stimulated by the presence of fatty acids (Abdel Motaal *et al.*, 2006). It is likely that this enzyme integrates these two signals and produces cAMP as the output signal, which then triggers an appropriate cellular response (Abdel Motaal *et al.*, 2006). A second adenylyl cyclase, Rv1264, is closely related to Rv2212 and was shown to bind oleic acid, and while the role of this fatty acid appears to be structural rather than regulatory, it cannot be excluded that it also serves as a functional signal in response to a changing lipid environment (Findeisen *et al.*, 2007). Further investigations into the sensing mechanisms involved in adaptation to different carbon sources are required to gain a better understanding of this crucial process in the physiology of mycobacteria.

10.1.2. Nitrogen source—The preferred nitrogen source for mycobacteria are amino acids, particularly asparagine, glutamate and aspartate, but they are also able to use inorganic sources such as ammonium salts (Ratledge, 1976). The uptake system for glutamate was found to be inducible by the presence of glutamate in the growth medium in *M. tuberculosis* and *M. smegmatis*, but constitutive in *M. fortuitum* and *M. phlei*(Lyon *et al.*, 1967). This suggests that a sensory mechanism exists in some species of mycobacteria to detect glutamate, but its nature has not been identified to date.

In the absence of other nitrogen sources, mycobacteria can assimilate ammonium, a process, which requires the enzymes glutamine synthetase (GS) and glutamate synthase. Uptake of ammonium in actinomycetes is via the AmtB transport system, and regulation of this transporter occurs posttranslationally via GlnK, which in turn is regulated through reversible adenylation by GlnD in response to nitrogen levels. This process has been studied in detail in *C. glutamicum* (Strosser *et al.*, 2004), but not yet in mycobacteria. The gene organization as an *amtB-glnK-glnD* operon is conserved within the Actinomycetales, and it is therefore likely that the mechanism of regulation is also conserved (Harper *et al.*, 2008).

In addition to the ammonium uptake system, the activity of GS is also subject to nitrogen-dependent regulation. In *S. coelicolor*, posttranslational control of GS activity is via reversible adenylation by GlnE (Fink *et al.*, 1999), and because GlnE is also present in the

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genome of *M. tuberculosis*(Cole *et al.*, 1998) regulation of GS activity in this bacterium is thought to involve the same mechanism (Harper *et al.*, 2008).

Transcriptional regulation of the genes involved in nitrogen metabolism, including *amtB* and *glnA* (encoding GS), in *Streptomyces coelicolor* requires the global regulator of nitrogen metabolism, GlnR (Tiffert *et al.*, 2008). Some evidence exists that this mechanism of regulation is conserved in many actinomycetes, including *M. tuberculosis* (Tiffert *et al.*, 2008). The primary sensor for the nitrogen status has not yet been identified in actinomycetes, nor is it known which molecule constitutes the signal, although intracellular concentrations of 2-oxoglutarate or ammonium ions appear to be likely candidates (Harper *et al.*, 2008).

10.1.3. Phosphate and sulphate (Inorganic anions)—Inorganic phosphate is the main source of phosphorus to mycobacteria, and genes involved in its uptake have been implicated in virulence of *M. tuberculosis* (Collins *et al.*, 2003; Peirs *et al.*, 2005). The two-component regulatory system responsible for phosphate limitation induced expression of high-affinity phosphate uptake systems and other genes involved in acquisition of phosphate was recently identified in *M. smegmatis* (Glover *et al.*, 2007). SenX3 is a membrane-bound histidine kinase which responds to low extracellular concentrations of phosphate by phosphorylation of the cognate response regulator, RegX3, leading to activation of gene expression. Expression of RegX3-dependent genes in *M. smegmatis* is deregulated in mutants lacking the high-affinity phosphate transport system Pst (Gebhard and Cook, 2008; Kriakov *et al.*, 2003), and it has been proposed that the Pst transporter constitutes the actual sensor of phosphate availability. Similar to the situation in *E. coli* (Wanner, 1996), mycobacterial SenX3 lacks an obvious extracellular loop and thus is probably unable to bind phosphate itself, but rather obtains this information indirectly via the Pst system (Glover *et al.*, 2007). A further protein, PhnF, is involved in regulation of phosphate transport in *M. smegmatis*. PhnF is a cytoplasmic protein and predicted to respond to a small soluble molecule present in the cell under phosphate-limited conditions, but the nature of this ligand has not been identified (Gebhard and Cook, 2008).

Expression of mycobacterial genes involved in assimilation and metabolism of sulphate is dependent on environmental signals such as sulfur limitation and oxidative stress as reviewed in Bhave *et al.* (2007) and Schelle and Bertozzi (2006). This regulation has been studied in some detail for key genes of sulphate assimilation, *cysD* and *cysNC*, of *M. tuberculosis* (Pinto *et al.*, 2004), but the sensing or regulatory mechanism involved remains unknown. One candidate for the control of sulfur-metabolism genes in response to environmental stimuli is the ECF sigma factor SigH of *M. tuberculosis*. Manganelli and colleagues were able to show that induction of several genes of cysteine biosynthesis in response to oxidative stress by diamide treatment was abolished in a SigH deletion mutant (Manganelli *et al.*, 2002).

10.1.4. Metals and trace elements (Inorganic cations)—Potassium is an important intracellular ion, largely due to its role in counteracting osmotic pressure. The high-affinity potassium transport system KdpFABC is therefore carefully regulated in bacteria in response to extracellular potassium concentration and osmotic pressure (Gassel and Altendorf, 2001). The *kdpFABC* operons of *M. tuberculosis* and *M. smegmatis* were found to be induced by low potassium concentrations, and the regulatory mechanisms have been studied (Steyn *et al.*, 2003). Expression of *M. tuberculosis kdpFABC* is controlled by the KdpDE two-component system. The KdpD sensor kinase interacts with two accessory membrane-proteins, LprF and LprJ, and because KdpD does not contain an obvious extracellular sensing domain, it is thought that these proteins modulate KdpD activity, presumably in response to the extracellular K^+ concentration (Steyn *et al.*, 2003).

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Iron is an essential component of several metabolic enzymes, but excess iron in the cell is harmful, due to the release of oxidative agents by the Fenton reaction. Its intracellular concentration therefore has to be very tightly controlled. This is reflected by the large number of genes (a total of 153 genes) differentially expressed in response to iron availability in *M. tuberculosis* (Rodriguez *et al.*, 2002). Several genes involved in iron acquisition have also been found to be induced by growth of *M. tuberculosis* in macrophages, suggesting the bacteria encounter conditions of iron limitation during infection (Gold *et al.*, 2001; Schnappinger *et al.*, 2003). In mycobacteria, iron-dependent gene expression is controlled by the DtxR homologue IdeR. DtxR-like proteins act as repressors of transcription when in complex with Fe(II), and such a mechanism has been shown for IdeR of *M. tuberculosis* (Pohl *et al.*, 1999; Schmitt *et al.*, 1995). Thus IdeR acts as a direct sensor of intracellular iron concentrations in mycobacteria, inducing proteins for iron storage and repressing proteins for iron acquisition under conditions of excess iron (Gold *et al.*, 2001; Rodriguez *et al.*, 2002). Curiously, *ideR* of *M. tuberculosis* is an essential gene (Rodriguez *et al.*, 2002), while that of *M. smegmatis* is dispensable (Dussurget *et al.*, 1996). A second DtxR homologue, SirR, exists in the genome of *M. tuberculosis* (Cole *et al.*, 1998), but this protein contains a sequence with similarity to manganese binding sites and has been proposed to play a role in manganese-responsive gene regulation (Rodriguez, 2006).

Copper is another metal which is required for the activity of some enzymes, yet can cause oxidative damage when present as free Cu(I) within the cell. Liu and colleagues identified an operon, the *cso* operon, in *M. tuberculosis*, which is induced at high extracellular copper concentrations (Liu *et al.*, 2007). One of the genes in this operon, *ctpV*, encodes a putative copper transporter and is thought to be required for detoxification of intracellular copper. It was shown that expression of the *cso* operon is regulated by the copper-sensor CsoR, encoded by the first gene of the *cso* operon. CsoR acts as a repressor in the absence of copper by binding to the *cso*-promoter. CsoR detects intracellular copper directly by binding Cu(I) ions, and in its Cu(I)-bound form is unable to bind to DNA, and repression of the *cso* operon is relieved (Liu *et al.*, 2007).

Regulation of zinc uptake in environmental and pathogenic mycobacteria occurs via yet another metal responsive transcriptional regulator, Zur (also named FurB). It is encoded in the Rv2358-*furB* operon in *M. smegmatis* and *M. tuberculosis*, and induced in response to high zinc concentrations (Milano *et al.*, 2004). This induction occurs via a second regulatory protein, encoded by Rv2358, which acts as a repressor in the absence of zinc and then repression is relieved when zinc is bound (Canneva *et al.*, 2005). Zur (FurB) itself also acts as a transcriptional repressor, but binds to the promoters of its target genes in the presence of Zn(II) ions (Lucarelli *et al.*, 2007; Maciag *et al.*, 2007). Microarray analysis of a *zur* mutant of *M. tuberculosis* together with DNA-binding assays revealed eight promoters (24 genes) that are directly controlled through Zur. Among these are several genes with putative functions in zinc uptake and it has been proposed that Zur is involved in regulation of zinc transport (Maciag *et al.*, 2007). Rv2358 and Zur likely constitute a zinc-responsive regulatory network of mycobacteria to ensure sufficient zinc uptake while avoiding the accumulation of toxic intracellular concentrations: At low extracellular zinc concentrations, transcription of Rv2358-*furB* is repressed by Rv2358 and any Zur proteins present in the cell are inactive, leading to expression of zinc-uptake systems. At high extracellular zinc concentrations, repression of *furB* transcription is relieved, Zur binds intracellular Zn(II) ions, represses transcription of zinc uptake and therefore prevents excess uptake.

10.2. Oxygen and redox state

Mycobacteria are obligate aerobes and as such have to possess mechanisms to detect the ambient oxygen tension to enable them to adapt to changes in oxygen availability by

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adjusting their metabolism accordingly. Furthermore, gradual depletion of oxygen has been implicated in entry of *M. tuberculosis* into non -replicating persistence and latency (Wayne and Sohaskey, 2001). The mechanisms by which mycobacteria sense oxygen have been extensively studied, and the two major sensory systems known to date, the DosT/DosS/DosR system and WhiB3, are discussed below.

10.2.1. The Dos-system—The dormancy survival, or Dos, system consists of the three proteins DosR (or DevR), DosS (or DevS) and DosT. DosR has been shown to act as a transcriptional regulator, which is responsible for the induction of nearly all hypoxia-induced genes of *M. tuberculosis*(Park *et al.*, 2003). The activity of DosR is regulated by the two sensor kinases, DosS and DosT, which phosphorylate DosR under conditions of low oxygen tension or in the presence of NO or CO (Kumar *et al.*, 2007; Roberts *et al.*, 2004; Sousa *et al.*, 2007). DosS and DosT both appear to contribute about equally to the activation of DosR in response to hypoxia. Single deletions of either *dosS* or *dosT* in *M. tuberculosis* H37Rv caused about a 45% decrease in activation of the hypoxia reporter gene *hspX*, while in a *dosS*/*dosT* double mutant induction of the *hspX* promoter was abolished completely (Roberts *et al.*, 2004). The Dos system is not restricted to pathogenic mycobacteria, because homologues of DosR and DosS were identified in *M. smegmatis* (Mayuri *et al.*, 2002), and an *M. smegmatis dosR* deletion mutant was impaired in survival of oxygen limited stationary phase (O'Toole *et al.*, 2003).

Two independent studies recently revealed the mechanism of activation of both DosT and DosS in response to oxygen, NO and CO. Both proteins contain one heme as a prosthetic group, and both proteins can bind oxygen, NO and CO (Kumar *et al.*, 2007; Sousa *et al.*, 2007). While DosT is relatively resistant to oxidation to the ferric form (Sousa *et al.*, 2007), DosS is rapidly converted to the met form (Fe^{3+}) when exposed to oxygen (Kumar *et al.*, 2007). The autokinase activity of DosS is drastically reduced when oxidized to met-DosS, and this finding led to the proposal that DosS acts as a redox sensor (Kumar *et al.*, 2007). This is consistent with the findings of Sousa and colleagues, who found only a six-fold difference in autokinase activity between oxy-DosS and deoxy-DosS (Sousa *et al.*, 2007). In contrast, the activity of DosT is regulated solely by its oxygenation state, where deoxy-DosT has about a 50 times higher autokinase activity than oxy-DosT (Sousa *et al.*, 2007). DosT is therefore thought to be an oxygen sensor, which is active in the absence of oxygen and looses its activity in the presence of oxygen (Kumar *et al.*, 2007; Sousa *et al.*, 2007). In addition to hypoxia, the Dos-regulon has also be shown to be activated by exposure of *M. tuberculosis* to NO or CO (Kumar *et al.*, 2008; Shiloh *et al.*, 2008; Voskuil *et al.*, 2003). An elegant study of the binding kinetics of oxygen, NO and CO to DosT and DosS have shown that this is most likely due to competition for binding between the ligands. The equilibrium dissociation constants (K_d) of NO and CO binding to DosT are 5000 times and 30 times lower, respectively, than that of oxygen binding (Sousa *et al.*, 2007). NO and CO, unlike oxygen, do not "switch off" DosT activity, but because of their much stronger binding displace oxygen from its binding site, thus simulating low oxygen tension and activating DosT autophosphorylation activity (Sousa *et al.*, 2007). A similar phenomenon was observed for DosS, where NO and CO appear to stabilize DosS in its active, ferrous form (Fe^{2+}), thus maintaining the active form of the protein even in the presence of oxygen (Kumar*et al.*, 2007).

10.2.2. WhiB3—Another protein, which has been implicated in sensing of oxygen and redox state by mycobacteria, is WhiB3. The *whiB3* gene was found to be strongly induced in *M. tuberculosis* during acute infection of mouse lungs and also during growth in resting bone marrow-derived macrophages, but repressed after INF-γ activation of the macrophages(Banaiee *et al.*, 2006). The authors hypothesized that this might be due to production of NO by activated macrophages, but were unable to identify an *in vitro* stimulus

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(NO exposure, oxidative stress, hypoxia or other stresses) leading to *whiB3* induction (Banaiee *et al.*, 2006). More recently, Singh and colleagues were able to show that WhiB3 contains a 4Fe-4S cluster, which can bind NO (Singh *et al.*, 2007). Furthermore, in the presence of oxygen, the WhiB3 $[4Fe\text{-}4S]^{2+}$ cluster is degraded first to a $[3Fe\text{-}4S]^{+}$ cluster, then a $[2Fe\text{ -}2S]^{2+}$ and subsequently lost altogether, in a mechanism reminiscent of the one found in the *E. coli* oxygen sensor FNR (Crack *et al.*, 2004; Singh *et al.*, 2007). Apo-WhiB3 was shown to have protein disulfide reductase activity, and it has been proposed that loss of the Fe-S cluster is required to gain this activity (Suhail Alam and Agrawal, 2008). WhiB3-mediated response to the presence of oxygen therefore may occur through direct control of the activity of metabolic proteins or through modification of transcriptional regulators (Suhail Alam and Agrawal, 2008). A role of WhiB3 in regulation of the transcriptional machinery in mycobacteria may be supported by the finding that WhiB3 interacts with the major sigma factor, SigA (RpoV) (Steyn *et al.*, 2002), but the effect of this interaction on SigA activity is not known, and further study is required to understand the precise role of WhiB3 in mediating any adaptation of mycobacteria to changes in oxygen tension.

10.2.3. Additional systems—While the Dos system and WhiB3 are the most studied regarding the perception of oxygen tension by mycobacteria, they are likely not the only systems used by these bacteria. For example, DosR of *M. tuberculosis* H37Rv was shown not to be strictly required for survival of hypoxia *in vitro* (Rustad *et al.*, 2008). The authors further found that expression of Dos regulon genes in response to hypoxia was transient, with expression of about half of the 50 DosR-dependent genes returning to baseline levels within 24 h of hypoxia. In contrast, a set of 230 genes was significantly up-regulated at four and seven days of hypoxia, but not initially, and were termed the enduring hypoxic response (EHR) (Rustad*et al.*, 2008). Induction of EHR was independent of DosR, suggesting that other sensory and regulatory mechanisms must exist to signal prolonged exposure to hypoxia. Strikingly, the EHR genes contained an unusually high number of regulatory genes (FurA, FurB, PhoP, three WhiB family members and two ECF sigma factors, SigH and SigE), but it is not yet known which of these, if any, are involved in entry into EHR (Rustad *et al.*, 2008). A study into the effect of addition of cAMP to growing cultures or *M. bovis* BCG or *M. tuberculosis* H37Rv found that the number of genes affected by cAMP was larger under low oxygen, CO_2 enriched conditions than under ambient air (Gazdik and McDonough, 2005). The authors proposed that cAMP may be used by mycobacteria as a signaling molecule in response to hypoxic conditions. However, the signal leading to cAMP synthesis or which adenylylcyclase might catalyse this reaction under hypoxia remains unknown.

10.3. Exogenous stress conditions

10.3.1. Oxidative stress—As obligate aerobes, mycobacteria are exposed to oxidative stress. Furthermore, the killing mechanisms of macrophages include production of reactive oxygen species, and the ability of pathogenic mycobacteria to respond to oxidative stress is thus essential for establishing an infection. In enteric bacteria, the oxidative stress response is mediated through the transcriptional regulator OxyR (Christman *et al.*, 1985; Tartaglia *et al.*, 1989). In most mycobacteria, with the exception of *M. leprae* and *M. avium*, the *oxyR* orthologous genes are inactivated by several mutations (Deretic *et al.*, 1995; Sherman *et al.*, 1995), and induction of an oxidative stress response must therefore be via a different mechanism. There appear to be considerable differences between saprophytic and pathogenic species, because *M. smegmatis* was found to mount a protective oxidative stress response, while *M. tuberculosis* and *M. avium* could not (Sherman *et al.*, 1995). Several studies have focused on how mycobacteria perceive and respond to reactive oxygen species, and a plethora of sensory and regulatory systems have been identified, confirming the importance of this stress condition to mycobacteria.

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A mutant of *M. smegmatis* in the iron-responsive regulator IdeR had an increased sensitivity to oxidative stress (Dussurget *et al.*, 1996), and this was shown to be due to reduced expression of catalase-peroxidase (KatG) and iron-dependent superoxide dismutase (SodA) (Dussurget *et al*, 1998). However, the molecular mechanism of *katG* and *sodA* regulation by IdeR in *M. smegmatis* remain to be discovered. Although an *ideR* mutant of *M. tuberculosis* H37Rv also showed an increase in sensitivity to oxidative stress, neither expression of *katG* and *sodA* nor the activities of the respective proteins was affected by the mutation (Rodriguez *et al.*, 2002). The role of IdeR in the oxidative stress response of *M. tuberculosis* therefore appears to differ from that in *M. smegmatis*. A further example of coupling the response to oxidative stress with iron metabolism in mycobacteria is regulation of *katG* by FurA, which is annotated as a ferric uptake regulator (Cole *et al.*, 1998), although a definite role for this protein in control of iron uptake in mycobacteria could not be shown (Pym *et al.*, 2001; Zahrt and Deretic, 2001). FurA is encoded by a gene immediately upstream of *katG* in all mycobacterial species tested, and was shown to be involved in the repression of *katG* but not *katE* or *ahpC* in *M. smegmatis* (Zahrt and Deretic, 2001). Similar results were obtained for *M. tuberculosis*, where it was shown that expression of the *furA-katG* operon, but not of *sodA* or *ahpC* was repressed by FurA (Pym *et al.*, 2001). The mechanism by which FurA detects oxidative stress, as well as how it represses transcription of *katG* and which other genes might be under its control is not yet known.

The protein WhiB4 of *M. tuberculosis* has also been implicated to play a role during oxidative stress (Alam *et al.*, 2007). Like WhiB3 discussed above, WhiB4 contains a [4Fe-4S] cluster, and this cluster is converted to a [2Fe-2S] cluster and subsequently lost from the protein upon exposure to oxygen or hydrogen peroxide. Upon loss of the Fe-S cluster, two intramolecular disulphide bonds are formed and WhiB4 gains protein disulphide reductase activity. This gain of activity under oxidative conditions led to the proposal that WhiB4, and possibly other WhiB proteins, play an important role in redox signaling during oxidative stress in *M. tuberculosis*. The effect of WhiB4 may be direct through reactivation of cellular proteins which have formed non-specific disulphides, or it is an indirect effect through modification of the activity of transcriptional regulators (Alam *et al.*, 2007).

Several alternative and ECF sigma factors have been shown to play a role in resistance of mycobacteria to oxidative stress. These include SigH (Manganelli *et al.*, 2002; Raman *et al.*, 2001) and SigJ (Hu *et al.*, 2004) in *M. tuberculosis*, and SigH (Fernandes *et al.*, 1999) and SigF (Gebhard *et al.*, 2008) in *M. smegmatis*. Of these, the role of *M. tuberculosis* SigH has been studied in most detail and it was shown that activity of the sigma factor is controlled by a redox-responsive anti-sigma factor, RshA (Song *et al.*, 2003). The gene arrangement of *sigH-rshA* is conserved in *M. tuberculosis*, *M. smegmatis* and *M. avium*. Binding of *M. tuberculosis* RshA to SigH occurs only under reducing conditions, with SigH being released and therefore active during oxidative stress (Song *et al.*, 2003).

10.3.2. Extracellular pH—Maintenance of a near-neutral internal pH despite changes in external pH is essential for all bacteria. Environmental mycobacteria are likely to encounter acidic pH in their soil habitat (Iivanainen *et al.*, 1999), and pathogenic mycobacteria are exposed to a drop in pH upon entry into the phagosome (Amer and Swanson, 2002). A reduction in external pH was shown to cause differential expression of a large number of genes in *M. tuberculosis* (Fisher *et al.*, 2002; Saviola *et al.*, 2003), and *M. smegmatis* and *M. bovis* BCG exhibited intracellular pH homeostasis over a large range of external pH values (Rao *et al.*, 2001). These data imply that mycobacteria are able to perceive the pH of their environment, and two adenylylcyclases have been suggested to fulfill that role in *M. tuberculosis*. Rv1264 has a 40-fold increased adenylylcyclase activity at pH 6 as compared to pH 8, and this pH-dependent activation is mediated by a pH-responsive catalytic domain (Tews *et al.*, 2005). In response to pH, the protein undergoes major conformational

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rearrangements and these may involve an unsaturated fatty acid as a structural element (Findeisen *et al.*, 2007; Tews *et al.*, 2005). In the closely related adenylylcyclase Rv2212, pH-sensing activity is strongly induced by binding of unsaturated fatty acids, but the mechanism of this effect is not fully understood (Abdel Motaal *et al.*, 2006). While both Rv1264 and Rv2212 have increased activity at acidic pH, the mechanism for activation appears to be different between the two proteins, because the pH response of Rv1264 is largely due to an increase of V_{max} (Tews *et al.*, 2005), while that of Rv2212 is due to an increase in substrate affinity for ATP (Abdel Motaal *et al.*, 2006).

10.3.3. Heat shock—The heat shock response of *M. tuberculosis* involves the up-regulation of over 100 genes, including those encoding the ECF sigma factors SigH and SigE, the general stress sigma factor SigB, and conserved heat shock proteins such as the Hsp60/GroE family and Hsp70 (DnaK) (Stewart *et al.*, 2002). Control of the heat shock response involves a network of overlapping regulatory circuits. SigH and SigE upregulate expression of many genes in response to elevated temperatures (Fernandes et al., 1999; Manganelli *et al.*, 2002; Manganelli *et al.*, 2001; Raman *et al.*, 2001), while HspR and HrcA act as transcriptional repressors (Stewart *et al.*, 2002). Furthermore, heat inducible expression of SigE is dependent on SigH, and expression of SigB depends on both SigE and SigH (Raman *et al.*, 2001). The mode of signal (i.e. heat) recognition by these regulators is not well understood, but evidence exists that the interaction between SigH and its anti-sigma factor RshA is disrupted by elevated temperatures (45°C to 55°C), leading to activation of SigH (Song*et al.*, 2003).

Curiously, *M. leprae*, which has a reduced ability to survive temperatures above 33°C, appears to lack a heat shock response. The key heat shock genes of *M. tuberculosis* are not induced by a shift from 33 °C to 37°C or 45°C in *M. leprae*, although functional copies of the heat shock regulators SigE, HspR and HcrA are present (Williams *et al.*, 2007). However, *sigH* of *M. leprae* is a pseudogene, and it is therefore likely that the lack of heat shock response in this bacterium is due to a lack of SigH-dependent induction of SigE and SigB (Williams *et al.*, 2007), further confirming the central role of this ECF sigma factor in the orchestration of a heat shock response in mycobacteria.

10.3.4. Entry into phagosome/macrophage—Due to the importance of this process for the virulence of *M. tuberculosis*, the reaction of the bacterium to entry into the phagosome has been the focus of many studies. At the same time it is extraordinarily difficult to dissect the various responses occurring, because there is no one stimulus associated with the transition to the intracellular environment. The bacteria will be exposed to a drop in pH, elevated temperatures, reactive oxygen and nitrogen species as well as a change in nutrient availability (Amer and Swanson, 2002; Schnappinger *et al.*, 2003). Several two-component regulatory systems have been implicated in being important for the adaptation to intracellular growth based on attenuation of corresponding mutants. However, it is difficult to ascertain whether the attenuation is due to a specific requirement of the regulatory system in the intraphagosomal environment or simply reflects a decrease in the bacteria's ability to cope with adverse conditions.

One of these two-component systems is MtrAB. The gene encoding the response regulator, *mtrA*, is essential in *M. tuberculosis* (Zahrt and Deretic, 2000). A transcriptional fusion of *M. tuberculosismtrA* to *gfp* is upregulated during growth of *M. bovis* BCG in murine macrophages as compared to *in vitro* growth (Via *et al.*, 1996), but constitutively expressed in *M. tuberculosis* grown *in vitro* or murine or human macrophages (Zahrt and Deretic, 2000), suggesting differences in *mtrA* expression between virulent and avirulent strains. Overexpression of MtrA in *M. tuberculosis* caused attenuation for growth in macrophages, mouse spleens and lungs and an impaired ability to block phagosome-lysosome fusion. This

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depended on the phosphorylation-competence of MtrA, suggesting that maintenance of a certain MtrA to phospho-MtrA ratio within the cell was essential for proliferation of *M. tuberculosis*(Asensio *et al.*, 2006). The expression of the essential replication initiation gene, *dnaA*, is controlled by MtrA, and it has been proposed that overexpression of *dnaA* by excess phospho-MtrA during infection could lead to the observed growth defect (Fol *et al.*, 2006). MtrB of *M. avium* has been proposed to play a role in regulation of cell surface proteins and permeability of the cell envelope (Cangelosi *et al.*, 2006). However, neither the environmental signal detected by MtrAB nor the complete MtrAB regulon have been identified to date.

The two-component system MprAB was identified as being required for virulence during the persistent stage of infection, and mutants in the response regulator gene, *mprA*, had altered growth characteristics in murine and human macrophages (Zahrt and Deretic, 2001; Zahrt *et al.*, 2003). As for MtrAB, there were distinct differences between virulent and avirulent strains, supporting a role for this system during infection: in *M. tuberculosis* H37Rv, *mprA* expression was not detected during growth in resting macrophages and an *mprA* mutant had increased intracellular survival compared to the wild-type (Zahrt and Deretic, 2001), whereas in *M. bovis* BCG, *mprA* was induced during intracellular growth and an *mprA* mutant was impaired in survival (Zahrt and Deretic, 2001; Zahrt *et al.*, 2003). Microarray analysis showed that the MprAB system regulates several sets of genes under different growth conditions, including normal growth and detergent stress (He *et al.*, 2006). Two of the genes under direct control of MprAB and induced by detergent stress are the general stress sigma factors, SigE and SigB (He *et al.*, 2006; Pang *et al.*, 2007), indicating the existence of a complex regulatory network, because expression of *sigB* and of *mprAB* itself is also controlled by SigE under these conditions (Manganelli *et al.*, 2001).

Mutants of *M. tuberculosis* in a further two -component regulatory system, the PhoPR system, were impaired in growth within macrophages and attenuated in virulence in mice (Perez *et al.*, 2001; Walters *et al.*, 2006). PhoP was shown to control expression of several genes involved in synthesis of complex cell wall lipids, some of which had previously been identified as virulence genes (Walters*et al.*, 2006). Because of its sequence similarity to the Mg^{2+}-responsive system PhoPQ of *Salmonella* (Lejona *et al.*, 2003), *M. tuberculosis* PhoPR was also thought to respond to Mg^{2+}limitation, and the *phoP* mutant indeed displayed an increased requirement for Mg^{2+}ions for growth (Walters *et al.*, 2006). However, no clear evidence is available that Mg^{2+} limitation constitutes the signal for PhoP-dependent gene expression, and it is rather thought that the structural alterations in the cell envelope necessitate Mg^{2+}ions for the stabilization of the mutants' cell wall (Walters *et al.*, 2006). The physiological signal for PhoPR therefore remains to be discovered.

The PrrAB system of *M. tuberculosis* was shown to be transiently required during early stages of infection in murine macrophages and this period coincided with induction of the *prrA*-promoter (Ewann *et al.*, 2002). Other systems that have been shown to affect the intracellular growth of *M. tuberculosis* include DosRS (Malhotra *et al.*, 2004), and SenX3-RegX3 (Parish *et al.*, 2003), which are discussed above. Further study is required to elucidate the role of these and other sensory systems during growth of mycobacteria within the host, and to establish precisely what the signal perceived by each of these systems is.

11. ENERGETICS OF MYCOBACTERIAL GROWTH

M. tuberculosis is a metabolically versatile bacterium able to oxidize a variety of carbon sources, including carbohydrates, fatty acids, TCA cycle intermediates and host lipids (e.g. cholesterol). A striking feature of *M. tuberculosis* metabolism is the ability of the bacterium

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to remain viable in the absence of cell growth and adapt to changing environments in the host. The mechanisms responsible for these adaptations are poorly understood.

11.1. Mycobacterial metabolism *in vitro*

A large body of detailed literature is available on the physiology of mycobacterial metabolism (Edson, 1951; Ramakrishnan *et al.*, 1972; Ratledge, 1976; Ratledge, 1982; Wheeler and Blanchard, 2005). During *in vitro* growth of *M. tuberculosis*, various carbon and energy sources feed into central metabolic pathways where they are oxidized to pyruvate and ultimately CO_2. In mycobacteria, the primary route of glucose conversion to pyruvate is the Embden-Meyerhof-Parnas (EMP) pathway. Approximately 70% of the glucose is directed down the EMP pathway and the remaining glucose (30%) flows into the pentose phosphate pathway to generate C_5 and C_4sugars and reducing power in the form of NADPH (Ratledge, 1976). No other pathways exist for sugar catabolism in pathogenic mycobacteria. The preferred carbon source for mycobacteria is glycerol and the metabolic route for its degradation is well established in these bacteria (for reviews see (Ratledge, 1976; Wheeler and Blanchard, 2005)). The metabolism of glycerol involves the phosphorylation of glycerol to form glycerol-3-phosphate via glycerol kinase, followed by the oxidation of this intermediate to dihydroxyacetone phosphate via glycerol-3-phosphate dehydrogenase. This triose phosphate is further metabolised via the EMP pathway and the Krebs cycle. *Mycobacterium bovis* lacks the ability to utilize glycerol unless pyruvate is provided, and this defect has been shown to be due to a single nucleotide polymorphism in the gene *pykA* encoding for pyruvate kinase (Keating *et al.*, 2005). The Krebs cycle in *M. tuberculosis* appears a typical because α-ketoglutarate dehydrogenase is not present and is replaced by the following reaction scheme: α-ketoglutarate is first converted to succinyl semialdehyde by a α-ketoglutarate decarboxylase and then oxidized to succinate by a succinyl semialdehyde dehydrogenase coupled to NADP reduction (Tian *et al.*, 2005).

In general, the complete oxidation of one mole of glycerol yields 7 moles of reduced pyridine nucleotide (NADH) or equivalent ($FADH_2$). Six moles of NADH are generated by glycerol-3-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate dehydrogenase, isocitrate dehydrogenase, succinyl semialdehyde dehydrogenase and malate dehydrogenase and one mole of $FADH_2$(via succinate dehydrogenase). At present, it has not been experimentally determined how much energy (e.g. moles of ATP) is produced per mole of glycerol utilized by mycobacterial species. Hypothetical calculations based on a mycobacterial respiratory chain that has three proton translocating sites when NADH is used as the endogenous electron donor (Prasada-Reddy *et al.*, 1975), and assuming one mole ATP is synthesized per three protons translocated by the ATP synthase would suggest that the oxidation of one mole NADH leads to the synthesis of 2 moles of ATP. Oxidation of one mole of $FADH_2$ would lead to the synthesis of approximately 1 mole of ATP. In addition, 3 moles of ATP (or an energetically equivalent nucleotide) are produced via substrate level phosphorylation (via phosphoglycerate kinase, pyruvate kinase and succinyl-CoA synthetase) and one of these is consumed by glycerol kinase. Based on these calculations, the theoretical ATP yield would be 15 moles of ATP produced per mole of glycerol consumed. In *E. coli* and *Bacillus megaterium*, the complete oxidation of one mole of glycerol yields 14 moles ofATP (Downs and Jones, 1975; Farmer and Jones, 1976).

The energetics of mycobacterial growth has been studied in glycerol-limited continuous culture (Beste *et al.*, 2005). In the slow growing *M. bovis* BCG, at both low (0.01 h^{-1}) and high (0.03 h^{-1}) dilution rates, the cell yield or $Y_{glycerol}$ was 27 g [dry weight] cells/mol glycerol utilized and 33 g [dry weight] cells/mol glycerol utilized respectively (Beste *et al.*, 2005). Under similar growth conditions in continuous culture, the fast growing non-pathogenic saprophyte *M. smegmatis* exhibits a $Y_{glycerol}$ of 30 to 40 g [dry weight] cells/mol glycerol utilized over the dilution rate range (0.02 to 0.15 h^{-1}) (S.L. Tran and G.M. Cook,

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unpublished data). The glycerol consumption rate for maintenance functions ($m_{glycerol}$) is 0.28 mmoles of glycerol/h/ g [dry weight] cells (S.L. Tran and G.M. Cook, unpublished data), a value that is comparable with *E. coli*. Beste *et al.* (2007b) have studied the effect of growth rate on gene expression of *M. bovis* BCG in carbon -limited continuous culture at dilution rates of 0.01 h^{-1}(t_d= 69 h) and 0.03 h^{-1}(t_d= 23 h). At low growth rate, there was a down regulation of genes involved in aerobic respiration, TCA cycle enzymes and the ATP synthase, despite the oxygen tension being maintained at 70–100 %, but consistent with a decreased oxygen demand due to a low growth rate. The authors reported that there was some overlap with the transcriptional profile of *M. bovis* BCG to growth rate and both Wayne's model of persistence (Muttucumaru *et al.*, 2004) and macrophage infection (Schnappinger *et al.*, 2003), butlittle correlation with the response of *M. tuberculosis* to oxygen limitation in chemostat culture (Bacon *et al.*, 2004). These data suggest that adaptation to slow growth rate in carbon-limited conditions is an important trigger for gene expression in both the Wayne model of persistence and growth of *M. tuberculosis* in macrophages (Beste *et al.*, 2007b).

11.2. Mycobacterial metabolism *in vivo*

The now widely accepted view for *in vivo* metabolism of *M. tuberculosis* is that the bacterium switches its intermediary metabolism from carbon sources such as glucose and glycerol to fatty acids and host lipids during the course of infection (Munoz-Elias and McKinney, 2005; Munoz-Elias and McKinney, 2006; Munoz-Elias *et al.*, 2005; Munoz-Elias *et al.*, 2006; Schnappinger *et al.*, 2003; Pandey and Sassetti, 2008). This concept was first supported by the sequencing of the *M. tuberculosis* genome, which revealed a large repertoire of genes for lipid degradation (Cole *et al.*, 1998). A central role for fatty acid metabolism is also implied by the considerable duplication of genes involved in lipid metabolism in mycobacterial genomes (Cole *et al.*, 1998; Cole *et al.*, 2001; Fleischmann *et al.*, 2002; Garnier *et al.*, 2003). Further studies have shown an induction of genes in the phagosomal environment that encode for enzymes that are required for the biochemical activation and β-oxidation of fatty acids (Schnappinger *et al.*, 2003). Breakdown products of fatty acids are further metabolised via the Krebs cycle and the carbon conserving (no loss of CO_2) glyoxylate shunt (McKinney *et al.*, 2000; Schnappinger *et al.*, 2003). *M. tuberculosis* has two copies of the gene (*icl1* and *icl2*) that encodes the enzyme isocitrate lyase, a key enzyme in the glyoxylate shunt that converts isocitrate to succinate and glyoxylate. Recent work has unequivocally shown that a double knockout mutant (*icl1 icl2*) of *M. tuberculosis* is unable to establish an infection in mice due to its inability to metabolise host-derived lipids (Munoz-Elias and McKinney, 2005). The methylcitrate cycle is important for the metabolism of propionyl-CoA in *M. tuberculosis*, an intermediate of odd chain number fatty acid breakdown, but paradoxically is not essential for persistence in mice (Munoz-Elias *et al.*, 2006).

Two recent studies have shown that cholesterol, a major component of host cell membranes, is essential for *M. tuberculosis* persistence in the lungs of chronically infected animals and for growth within the IFN-γ-activated macrophages that predominate at this stage of infection (Gatfield and Pieters, 2000; Pandey and Sassetti, 2008). Pandey and Sassetti (2008) demonstrate that both *M. tuberculosis* and *M. smegmatis* utilize cholesterol, but the mechanism of energy generation during growth on cholesterol is unknown. Kendall *et al.* (2007) have identified a transcriptional repressor, KstR, that controls the expression of a significant number of genes involved in lipid metabolism in both *M. tuberculosis* and *M. smegmatis*.

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11.3. Recycling of reducing equivalents and electron transport

A crucial feature in the adaptation of any bacterium to alternative energy sources and changing environmental parameters (e.g. oxygen tension) is the balance of oxidative and reductive reactions in the metabolic scheme. During mycobacterial growth, both *in vitro* and *in vivo*, substrate oxidation leads to the formation of reduced cytoplasmic electron carriers, e.g. NADH, $FADH_2$ or ferredoxin. The oxidation of NADH or equivalent by aerobicbacteria is critical for continuous metabolic flux, and in the absence of a fermentative metabolism, NADH oxidation will be carried out primarily by membrane-bound NADH dehydrogenases. NADH dehydrogenase is the first component of the respiratory chain and transfers electrons from NADH to quinones (e.g. ubiquinone or menaquinone). Weinstein *et al.* (2005) have identified genes for two classes of NADH:menaquinone oxido reductases in the genome of *M. tuberculosis*. NDH-1 is encoded by the *nuoABCDEFGHIJKLMN* operon and as this dehydrogenase transfers electrons to menaquinone, it conserves energy by translocating protons across the membrane to generate a proton motive force ($\Delta\mu_H+$) (Fig. 7). The second class is NDH-2, a non-proton translocating NADH dehydrogenase that does not conserve energy and is present in two copies (*ndh* and *ndhA*) in *M. tuberculosis*(Weinstein *et al.*, 2005). Mutagenesis studies have established that both NDH-1 and *ndhA* are dispensable for growth *in vitro* (Sassetti *et al.*, 2003), but the lack of a viable strain with a disrupted or deleted *ndh* suggests that it is essential for growth despite the presence of *ndhA*(McAdam *et al.*, 2002; Sassetti *et al.*, 2003; Weinstein *et al.*, 2005). Furthermore, deleterious point mutations in *ndh* of *M. smegmatis* are pleiotropic, conferring temperature -sensitive growth arrest and multiple amino acid auxotrophy. Some mutants also have a 25-fold reduced NADH dehydrogenase activity, implying NDH-2 is the primary enzyme responsible for NADH oxidation and it is essential for the viability of *M. smegmatis*(Miesel *et al.*, 1998; Vilcheze *et al.*, 2005).

In *M. tuberculosis*, no energetic role for the non-essential type I NADH:menaquinone oxido reductases has been established and the persistence of *M. tuberculosis* in an *in vitro* Wayne model was shown not to be compromised in a *nuo* operon deletion mutant (Rao *et al.*, 2008). Velmurugan *et al.* (2007) have demonstrated a role for *nuo* (i.e. the NuoG subunit) in the ability of *M. tuberculosis* to inhibit macrophage apoptosis. The *nuo* operon has been lost from the genome of *M. leprae* with a single *nuoN* pseudogene remaining (Cole *et al.*, 2001). *M. smegmatis* also contains genes for a type I NADH:menaquinone oxido reductases (viz. *nuoA-N*), but enzyme assays for NDH-I activity in *M. smegmatis* failed to detect the enzyme suggesting it is not expressed (Miesel *et al.*, 1998). Several studies have reported that *nuo* is down-regulated in *M. tuberculosis* during mouse lung infection (Shi *et al.*, 2005), survival in macrophages (Schnappinger *et al.*, 2003), in both NRP-1 (1% oxygen saturation) and NRP-2 (0.06% oxygen saturation) relative to aerated mid-log growth (Shi *et al.*, 2005), and upon starvation *in vitro* (Betts *et al.*, 2002). The transcription of *ndh* is also down-regulated in *M. tuberculosis* during mouse lung infection, but transcript levels for *ndh* peak (are induced) during NRP-2 *in vitro* demonstrating that the pattern of *ndh* regulation is different between *in vivo* and *in vitro* conditions (Schnappinger *et al.*, 2003). These data are in contrast to *E. coli* where NuoA-N (NDH-1) is usually associated with anaerobic respiratory pathways (e.g. fumarate) and non-coupling dehydrogenases such as NDH-2 are synthesized aerobically (Unden and Bongaerts, 1997). Moreover, *E. coli* mutants lacking NDH -I have a competitive disadvantage in stationary phase (Zambrano and Kolter, 1993). Some interesting questions arise from these observations. The first is “why do mycobacteria use type II NADH dehydrogenases to recycle NADH when they could continue to use the energy conserving ($\Delta\mu_H+$ generating) NDH-1?” One potential explanation is that because NDH-2 are non-proton translocating, they will not be impeded by a high $\Delta\mu_H+$ which could ultimately slow down glycolytic flux due to back pressure on the system. This mechanism is akin to a “relief valve” that would allow for a higher metabolic flux and ultimately higher

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rates of ATP synthesis at the expenses of low energetic efficiency of the respiratory chain. Secondly, why is *ndh* an essential gene when mycobacteria could also use *ndh2* or *nuo*? The fact that *ndh* is essential implies that mycobacteria do not have another mechanism to recycle NADH during normal aerobic growth. Alternatively, this is the only NADH dehydrogenase that is operating under these growth conditions and the activity of this enzyme and subsequent electron transfer (and proton translocation) to oxygen is what fuels the essential F_1F_o-ATP synthase of mycobacteria (Tran and Cook, 2005).

The mechanisms used by mycobacteria to recycle NADH under either hypoxia or anaerobic conditions remain unknown. Compounds that target either the F_1F_o-ATP synthase or NDH-2 are bactericidal towards hypoxic non-replicating *M. tuberculosis* suggesting that the respiratory chain is essential for the recycling of NADH under these conditions (Rao *et al.*, 2008).

11.4. Aerobic respiratory pathways in mycobacteria

During aerobic respiration, electrons from the oxidation of either NADH (via NADH:menaquinone oxidoreductases) or succinate (via succinate dehydrogenase *sdhABCD*) flow into the menaquinone$_{(oxid)}$-menaquinol$_{(red)}$pool (Fig. 7). In mycobacteria, menaquinol can transfer electrons either directly to a cytochrome *bd*-type menaquinol oxidase (encoded by *cydABCD*) or through a cytochrome *c* pathway (Boshoff and Barry, 2005; Kana *et al.*, 2001; Matsoso *et al.*, 2005). The cytochrome *bd* branch is the bioenergetically less efficient branch (non-proton translocating) and is synthesized at low oxygen tensions (Kana *et al.*, 2001). The cytochrome *c* pathway consists of a menaquinol-cytochrome *c* oxidoreductase termed the bc_1complex (encoded by the *qcrCAB* operon) and an aa_3-type cytochrome *c* oxidase (encoded by *ctaBCDE*) belonging tothe haem-copper respiratory oxidase family (Boshoff and Barry, 2005; Matsoso *et al.*, 2005). The cytochrome *c* oxidase functions as a proton pump and may form a "supercomplex" with menaquinol cytochrome *c* oxidoreductase (Matsoso *et al.*, 2005). Support for this hypothesis comesfrom the observation that such supercomplexes have been reported in other actinomycetes such as *C. glutamicum*(Niebisch and Bott, 2003). In addition to cytochrome *bd* and an aa_3-type cytochrome *c* oxidase, the *M. smegmatis* respiratory chain has been proposed to contain a third possible respiratory branch terminating in the YthAB (*bd*-type) menaquinol oxidase (Kana *et al.*, 2001). The bc_1-aa_3 pathway is the major respiratory route in mycobacteria under standard aerobic culturing conditions (Matsoso *et al.*, 2005). Matsoso *et al.* (2005) have demonstrated that disruption of this pathway in *M. smegmatis* is accompanied by a constitutive upregulation of the cytochrome *bd*-type menaquinol oxidase. In *M. tuberculosis*, the bc_1-aa_3 pathway is essential for growth suggesting an inability of this bacterium to adapt in a manner analogous to *M. smegmatis*. The aa_3 branch is also proposed to contain two *ctaD* alleles in *M. smegmatis* vers us the one in *M. tuberculosis*, suggesting alternate isoforms of cytochrome *c* oxidase in *M. smegmatis*(Kana *et al.*, 2001).

11.5. Generation of an electrochemical gradient of protons ($\Delta\mu_H+$) in mycobacteria

When growing aerobically at an external pH of 7.0, *M. smegmatis* and *M. bovis* BCG generate an $\Delta\mu_H+$ of approximately −180 mV which appears to be of a significant magnitude to drive proton-coupled bioenergetic processes (eg. ATP synthesis, solute transport, etc) (Rao *et al.*, 2001). Growth of mycobacteria is sensitive tothe electrogenic proton translocator carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) demonstrating that a $\Delta\mu_H+$ is indispensable for normal growth. Moreover, growth is inhibited by the F-type ATP synthase inhibitor *NN*′,-dicyclohexylcarbodiimide (DCCD) further supporting the role of the $\Delta\mu_H+$ in driving ATP synthesis by the membrane-bound F_1F_O-ATP synthase. While proton translocation via the respiratory chain generates the $\Delta\mu_H+$ during respiration with oxygen as the terminal electron acceptor, it is not clear how the $\Delta\mu_H+$ is established in the

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absence of oxygen under anaerobic growth conditions. Anaerobic bacteria are able to generate a significant $\Delta\mu_H+$ (−100 mV) using their membrane-bound F_1F_o-ATP synthase in the ATP hydrolysis direction (Dimroth and Cook, 2004). The ATPase activity (proton pumping) of the enzyme is fuelled by ATP produced by substrate level phosphorylation. The F_1F_O-ATP synthase of *M. tuberculosis* appears to have latent ATPase activity when purified suggesting that the enzyme may not function in the ATP hydrolysis direction (Higashi *et al.*, 1975). Whether the enzyme is also latent in actively growing cells is not known and therefore the potential exists for this enzyme to function as a primary proton pump in the absence of oxygen and a functional respiratory chain to generate the $\Delta\mu_H+$. To our knowledge, the effect of DCCD on anaerobic cultures of mycobacteria has not been determined to address this hypothesis. Rao *et al.* (2008) have reported that hypoxic, non -replicating *M. tuberculosis* generate a total proton motive force of −113 mV, −73 mV of electrical component ($\Delta\psi$) and −41 mV of ZΔpH. The addition of thioridazine, a compound that targets NDH-2, results in dissipation of the electrical potential and significant cell death suggesting that NADH is an important electron donor for the generation of the $\Delta\psi$ under hypoxic conditions. The addition of R207910 (TMC207), a specific inhibitor of the F_1F_o-ATP synthase was bactericidal against hypoxic, non-replicating *M. tuberculosis*, but had no effect on the $\Delta\psi$ (Rao*et al.*, 2008), an observation consistent with the latent ATPase activity of this enzyme (see above).

A potential solution to the generation of a $\Delta\mu_H+$ under hypoxia is the use of a respiratory nitrate reductase (Fig. 7). Breakdown of nitric oxide in mammalian tissue would provide a source of nitrate that could be used as an alternative electron acceptor. A transport system for the exchange of nitrate and nitrite into and out of the cell are present in the genome of *M. tuberculosis* (e.g. NarK2) (Cole, 1998). *M. tuberculosis* contains genes (*narGHJI*) that encode for a putative membrane-bound molybdenum-containing nitrate reductase complex similar to the corresponding *narGHJI* operon of *E. coli*. Moreover, the *narGHJI* operon of *M. tuberculosis* is able to functionally complement a *nar* mutant of *E. coli* to grow on glycerol and reduce nitrate anaerobically (Sohaskey and Wayne, 2003). Importantly however, the expression of *narGHJI* operon in *M.tuberculosis* is not upregulated in response to either hypoxia or stationary phase (Sohaskey and Wayne, 2003). Furthermore, Sohaskey and Wayne (2003) demonstrate that overexpression of recombinant *M. tuberculosis* nitrate reductase in either *M. tuberculosis* or *M. smegmatis* (low nitrate reductase) does not confer the ability of these cells to grow anaerobically ie. no growth of either species is observed with nitrate anaerobically even though the nitrate reductase activity of whole cells increases (Sohaskey and Wayne, 2003). The genome of *M. tuberculosis* also lacks orthologs of FNR which, in combination with NarL, are responsible for the transcriptional activation of the *narGHJI* operon anaerobically in *E. coli* (Unden and Bongaerts, 1997). A putative NarL (*Rv0884c*) has been indentifed in *M. tuberculosis* but the promoter of the *narGHJI* lacks consensus -like binding sites for this regulatory protein. Based on these observations, it is apparent that this enzyme does not support anaerobic growth of mycobacteria and therefore the role of this enzyme in the physiology of mycobacteria is unclear. Given the proposed membrane-bound location of the enzyme and the proton-pumping activity of the *E. coli* enzyme, perhaps the primary role of the mycobacterial enzyme is to generate a $\Delta\mu_H+$ when the concentration of oxygen is low, and hence its activity increases but not its expression. An alternative role for nitrate reductase may be maintaining the redox balance of the cell during conditions of hypoxia. Sohaskey (2008) has reported that exogenously supplied nitrate has no effect on long-term persistence during gradual oxygen depletion, but played an important role during rapid adaptation to hypoxia (< 18 h). This effect required a functional nitrate reductase, suggesting that nitrate reduction may play a role in protecting cells during sudden changes in oxygen concentration leading to disruption of aerobic respiration. Sohaskey (2005) proposes a role for NarK2 in sensing the redox state of the cell such that nitrate is transported into the cell under reducing, but not oxidizing conditions.

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The role of endogenous electron acceptors may also fuel the generation of a membrane potential ($\Delta\psi$) in the absence of exogenous acceptors. Mycobacteria utilize menaquinone/menaquinol as a conduit between electron-donating and -accepting reactions. Menaquinone has a lower midpoint redox potential (E_m= −74 mV) compared to ubiquinone (E_m= +113 mV) and is ideally poised to donate electrons to fumarate during anaerobic conditions (Cecchini *et al.*, 2002). Fumarate reductase (encoded by *frdABCD*) is present in *M. tuberculosis* and has been shown to be upregulated during carbon starvation and oxygen depletion (Betts *et al.*, 2002) and in macrophages (Schnappinger *et al.*, 2003), suggesting a role for this enzyme in persistence (Fig. 7). A recent study suggests that fumarate may be an important endogenous electron acceptor for energy production and maintenance of redox balance (oxidation of NADH to NAD^+) in hypoxic nonreplicating mycobacteria, but this remains to be experimentally validated (Rao *et al.*, 2008). Interestingly, the use of fumarate as an electron acceptor in *E. coli* requires complex I, and expression of the *nuo* operon is stimulated by the presence of fumarate (Unden and Bongaerts, 1997).

11.6. ATP synthesis by mycobacteria

In *M. tuberculosis*, ATP is synthesized via substrate level phosphorylation and oxidative phosphorylation using the membrane-bound F_1F_o-ATP synthases (encoded by the *atpBEFHAGDC* operon). No *atpI* gene is present in the operon, but an ORF (i.e. Rv1303) potentially may play this role being located immediately upstream of the *atp* locus. The F_1F_o-ATP synthase catalyzes ATP synthesis by utilizing the electrochemical gradient of protons to generate ATP from ADP and inorganic phosphate (P_i) and operates under conditions of a high $\Delta\mu_H$+ and low intracellular ATP. The enzyme is also capable of working as an ATPase, hydrolyzing ATP to pump protons from the cytoplasm to the outside of the cell and operates under conditions of high intracellular ATP and an overall low $\Delta\mu_H$+. In *M. smegmatis*, the *atp* operon has been shown to be constitutively expressed and an increase in expression is noted upon growth with non-fermentable substrates that are strictly coupled to oxidative phosphorylation (e.g. succinate) compared to fermentable carbon sources like glucose (Tran and Cook, 2005). The F_1F_o-ATP synthase in *M. tuberculosis* and *M. smegmatis* has been shown to be essential for growth (Sassetti *et al.*, 2003; Tran and Cook, 2005). In other bacteria, the F_1F_o-ATP synthase has been shown to be dispensable for growth on fermentable carbon sources (Friedl *et al.*, 1983; Santana *et al.*, 1994), where increased glycolytic flux can compensate for the loss of oxidative phosphorylation. This strategy does not appear to be exploited by *M. smegmatis*: the F_1F_o-ATP synthase is essential for growth even on fermentable substrates, suggesting that ATP production from substrate level phosphorylation alone, despite increased glycolytic flux, may be insufficient to sustain growth of these bacteria. This would be reflected by an extraordinarily high value for the amount of ATP required to synthesize a mycobacterial cell, a possibility that requires further investigation. Alternatively, or in conjunction with a high ATP demand for growth, the ATP synthase may be an obligatory requirement for the oxidation of NADH by providing a sink for translocated protons during NADH oxidation coupled to oxygen reduction. Such strict coupling would imply that mycobacteria do not support uncoupled respiration, either they lack a conduit for proton re-entry in the absence of the F_1F_o-ATP synthase or they are unable to adjust the proton permeability of the cytoplasmic membrane to allow a futile cycle of protons to operate. In this context, the cytoplasmic membrane of *M. smegmatis* is extremely impermeable to protons (Tran *et al.*, 2005).

Several new anti-tubercular drugs have recently been reported (Andries *et al.*, 2005; Weinstein *et al.*, 2005). It is particularly noteworthy that both classes of drugs target oxidative phosphorylation in mycobacteria. The first class, diarylquinolines, have beenshown to target the F_1F_o-ATP synthase to inhibit ATP synthesis by the enzyme

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(Andries *et al.*, 2005; Koul *et al.*, 2007). Given the essential role of this enzyme for mycobacterial growth (Tran and Cook, 2005), even on fermentable carbon sources, the ATP synthase would appear to represent a valid target. Genome sequencing of both *M. tuberculosis* and *M. smegmatis* mutants that are resistant to diarylquinolines (i.e. R207910) revealed that the target of these compounds is the oligomeric *c* ring (encoded by *atpE*) of the enzyme. Purified *c* ring from *M. smegmatis* binds R207910 with a K_D of 500 nm, and modelling studies suggest that R207910 blocks proton transfer by the enzyme (de Jonge *et al.*, 2007). The second class, phenothiazine analogs, target the NADH: menaquinone oxidoreductase (Weinstein *et al.*, 2005) suggesting that NADH oxidation via aerobic respiration coupled to oxidative phosphorylation is essential for the growth of mycobacteria.

11.7. Other mechanisms to recycle NADH during hypoxia

Hydrogenases are found in a wide variety of microorganisms and catalyse the reversible conversion of $H_2$to $2H^+$and $2e^-$ thus enabling the bacterium to utilize H_2 as a source of reducing equivalents. Conversely, some bacteria use the enzyme to reduce protons to H_2 thereby releasing the reducing equivalents. Hydrogen evolution is a mechanism commonly employed by anaerobic bacteria to recycle reducing equivalents obtained from anaerobic degradation of organic substrates. However, even strictly aerobic bacteria have been shown to produce H_2 under anaerobic conditions (Kuhn *et al.*, 1984). It has been shown that *M. smegmatis,* among other mycobacterial species, can oxidize molecular hydrogen in the presence of carbon monoxide, implying that *M. smegmatis* expresses a functional hydrogenase (King, 2003). No studies have reported the ability of mycobacteria to produce hydrogen, but analysis of the *M. tuberculosis* genome reveals the presence of a gene cluster (viz. Rv0082 to Rv0087) encoding components of a putative hydrogenase and formate hydrogen lyase that is upregulated during infection of human macrophage-like THP-1 cells (Fontan *et al.*, 2008). Moreover, the transcription of the early genes of the *hycP/hycQ* containing operon was shown to be upregulated during anaerobic adaptation in several studies (Bacon *et al.*, 2004; Park *et al.*, 2003; Sherman *et al.*, 2001; Voskuil *et al.*, 2003; Voskuil *et al.*, 2004). The putative hydrogenase gene cluster of *M. tuberculosis* shows homology to components of hydrogenase 4 and 3 complex of *Escherichia coli*, the latter of which has been shown to catalyze hydrogen evolution at acidic pH (Mnatsakanyan *et al.*, 2004). It is tempting to propose that the acidic, hypoxic and lipid-rich environment in the macrophages might require the expression of a hydrogenase complex in *M.tuberculosis* to help with the recycling of reducing equivalents under these conditions. The requirement for a low potential redox carrier in hydrogen production could be satisfied by reactions that are coupled to ferredoxins and not NADH. For example, *M. tuberculosis* contains a putative pyruvate:ferredoxin oxidoreductase (*Rv2454c/Rv2455c*) that would catalyse the oxidation of pyruvate to acetyl-CoA with reduced ferredoxins acting as the electron donor for hydrogen production. The role of hydrogen in the cycling of reducing equivalents in mycobacteria during hypoxia warrants investigation.

11.8. Control of energy generation in mycobacteria

In bacteria there is often a trade off between obtaining the maximum energy yield from a substrate and the maximum flux (rate of ATP production) or growth rate (Pfeiffer *et al.*, 2001). A cell that uses a pathway with a high yield and low rate (e.g. respirers) can produce more ATP from a given amount of substrate compared to a bacterium that produces ATP at a higher rate but a lower yield (e.g. fermenters). The growth yield of bacterial cultures has been used to estimate the efficiency of energy generation during respiration or fermentation. Mycobacteria would appear to adopt the first strategy where the cells grow slowly using oxidative phosphorylation to generate large amounts of ATP at a slow rate. Furthermore, this strategy appears to be employed by both fast growing and slow growing mycobacteria;

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the growth yield on glycerol is comparable between *M. smegmatis* and *M. bovis* BCG at similar growth rates (see above).

In order for mycobacteria to utilize oxygen efficiently and obtain the maximum growth yield on a particular carbon and energy source, there must be co-ordinate regulation of terminal respiratory oxidase expression. For example, *In E. coli* cytochrome *bo* (K_m for oxygen in the micromolar range) and cytochrome *bd*($K_m$for oxygen in the nanomolar range) (D'Mello *et al.*, 1995; D'Mello *et al.*, 1996) are coordinately regulated by the ArcBA system and FNR (Cotter *et al.*, 1997). Cytochrome *bo* is synthesized at high oxygen tension (optimal between 15 to 100% air saturation) and repressed as the oxygen concentrations decreases (Tseng *et al.*, 1994). This coincides with the induction of cytochrome *bd* at 7% air saturation, which is turned off (FNR-mediated repression) once the cells enter anaerobiosis (Cotter *et al.*, 1997; Tseng *et al.*, 1994). *E. coli* also uses non -coupling dehydrogenases (NDH-2) during aerobic growth that allow a fast metabolic flux (fast growth rate) and switches to coupling dehydrogenases (NDH-1) during anaerobic growth with fumarate (Unden and Bongaerts, 1997).

Mycobacteria also adopt regulation of oxidase expression to match oxygen supply. Under conditions of low oxygen tension (ca. 1 % air saturation), cytochrome *bd* is induced in *M. smegmatis* as the transition to anaerobiosis is approached (Kana *et al.*, 2001), a value that is 10-fold lower than that observed in *E. coli* (ca. 10% air saturation). In *M. tuberculosis*, cytochrome *bd* is upregulated in the early stages of NRP-1 (i.e. decreasing oxygen) (Voskuil *et al.*, 2004). A strategy that appears to be invoked by mycobacteria is one of down-regulation or a slowing of metabolism as cells enter NRP-1 and NRP-2. Transcriptional analysis of *M. tuberculosis* in the macrophage (phagosomal environment) has revealed that NDH-1, menaquinol-cytochrome *c* oxidoreductase and the ATP synthase are all down regulated when compared to cells growing exponentially suggesting the reduced need for energy generation during bacteriostasis i.e., the growth state of intraphagosomal *M. tuberculosis*(Schnappinger *et al.*, 2003). Consistent with these observations is the repression of these operons during starvation. In contrast, fumarate reductase, nitrate reductase and NDH-2 are all upregulated under these conditions (Schnappinger *et al.*, 2003). Whilst these proteins do not appear to contribute to increased energy production, it has been suggested that they may play a pivotal role in the recycling of NAD^+ as a result of β-oxidation of fatty acids (Schnappinger *et al.*, 2003). Furthermore, the induction of *ald*, encoding L-alanine dehydrogenase may also contribute to the recycling of NAD^+ through the reductive amination of pyruvate to alanine and subsequent oxidation of NADH.

While various components of the oxidative phosphorylation machinery are down-regulated (e.g. ATP synthase) during persistence or dormancy, this does not mean they are entirely absent. Koul *et al.* (2008) demonstrate that R207910 kills dormant *M. tuberculosis* as effectively as aerobically grown *M. tuberculosis* with the same target specificity. Moreover, the authors show that dormant *M. tuberculosis* do indeed have residual ATP synthase activity.

12. SYSTEMS BIOLOGY AND THE MYCOBACTERIAL CELL

Molecular biology, biochemistry and genetics have proved powerful disciplines in uncovering the individual functions of biomolecules and their interactions. However, whilst these traditional methods provide insight into the understanding of what constitutes a biological system, there is an increasing need to elucidate the overall working of a biological system (Kitano 2001; Kitano 2002). In this regard, mathematical models and computational analyses are required to attain such system level understanding (Kitano 2002; Stelling 2004). Mathematical models quantitatively and dynamically describe molecular interactions in

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biological networks and enable simulations of complex biological behaviour. Through such simulations, the ultimate goal of systems biology -understanding how global biological behaviour (e.g. a phenotype) emerges from the local interactions of molecular components (Kitano 2002) can be obtained.

A systems biology approach encompasses three main steps:

- **i.** Large-scale-omics measurement techniques (such as transcriptomics, proteomics, metabolomics, etc.) to provide quantitative experimental data on component concentrations.
- **ii.** In order to extract biological insight from these data, due to their size and the underlying complexity, computational tools need to be utilized. This step of computational analysis of large-scale data is typically referred to as "top-down" systems biology.
- **iii.** The knowledge about molecular components and interactions between those components does not allow for a system understanding *per se*. The "bottom-up" approach of systems biology is concerned with integrating the knowledge about the molecular interactions in a quantitative manner by means of mathematical models. Computational analysis of these models (i.e. through simulations) then leads to the envisioned system understanding.

In contrast to the field of *E. coli* or *S. cerevisiae*, little work along these lines has been performed with species of mycobacteria. In the following section, we will review the systems biology work that has been done to date in the mycobacterial field.

12.1. Large-scale omics analyses and computational analyses of such data

12.1.1. Transcriptomics—Transcriptomics using microarray and quantitative PCR (qPCR) is the experimental analysis tool most commonly applied for studies with *M. tuberculosis*. Three recent reviews comprehensively summarize the transcriptome studies published until 2007 (Bacon and Marsh, 2007; Kendall *et al.*, 2004; Waddell and Butcher, 2007). The majority of microarray data have been derived from batch cultures with low oxygen tension or nutrient starvation or from *ex vivo* macrophage mouse models. Only a few studies are available where mycobacteria were grown under more defined conditions like continuous culture. In macrophage or mouse model studies, the amount of bacterial mRNA is the limiting factor due to the low bacterial numbers in such systems. However, in a recent study, mycobacterial RNA was reproducibly amplified from as little as 5 ng total RNA (equivalent to 2×10^5 bacilli) (Waddell *et al.*, 2008). This opens up new possibilities for *in vivo* large -scale transcriptomics studies.

The analysis of microarray data is usually limited to extracting the significantly up-or down -regulated genes and gene clusters, classification into functional categories and, less often, superimposition onto metabolic pathways (Schnappinger *et al.*, 2003). The latter method is most promising for the understanding of the metabolic state of *M. tuberculosisin vivo* (Bacon and Marsh, 2007). Some studies also provide a meta-analysis, revealing regulons and gene clusters, which are similarly regulated under different conditions (Beste *et al.*, 2007b; Kendall *et al.*, 2004; Voskuil *et al.*, 2004). Beste and colleagues (2007a; 2007b) have used microarray data to validate an *in silico* genome scale -network model (see below).

A prerequisite for the use of transcriptional data in a systems biology approach is an exact knowledge of the growth parameters. However, in macrophage and *in vivo* studies, the composition and concentration of nutrients and the availability of terminal electron acceptors like oxygen or nitrate are difficult to assess. Furthermore, in the majority of gene expression studies, the control culture is growing at a different growth rate than the test

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culture. This difference alone can account for a different pattern of gene expression being observed (Beste *et al.*, 2007b; Ishii *et al.*, 2007). More defined *in vitro* approaches like continuous cultivation (chemostat), where bacteria can be grown on minimal medium in a carefully controlled environment, can help to dissect the complex *in vivo* environment (Bacon *et al.*, 2007; Bacon *et al.*, 2004; Beste *et al.*, 2007b). It will be important to use gene expression data from experiments with well-defined growth and environmental parameters (from *in vitro* approaches) to feed into the *in silico* models. *In silico* predictions can then be validated using *in vivo* data.

12.1.2. Proteomics—In order to gain a greater insight into mycobacterial physiology in reponse to the environment, the measurement of protein expression is an essential prerequisite. Many proteins are regulated post-transcriptionally and, therefore, transcriptome studies cannot account for these changes. Shotgun proteomics and 2D-Gel analysis are tools that can complement and extend our knowledge gained from DNA microarray analysis. Several studies with mycobacteria have been performed (Cho *et al.*, 2006; Jungblut *etal.*, 1999; Radosevich *et al.*, 2007). Recently, a shotgun protein expression analysis of *Mycobacterium smegmatis* challenged with three different anti-tubercular antibiotics revealed drug-specific changes in metabolic pathways as well as putative drug targets (Wang and Marcotte, 2008). Proteomics though, is still limited to *in vitro* studies because *in vivo* experiments do not yield enough material for a global protein analysis. Nevertheless, as mentioned above, well-defined *in vitro* studies are valuable for the construction and iterative improvement of *in silico* models.

12.1.3. Other omics—A global lipid profile (i.e. lipidomics) of *M. tuberculosis* has been performed by Jain *et al.* (2007). However, other omic techniques that are widely used with unicellular model organisms such as metabolomics (Dunn *et al.*, 2005; van der Werf *et al.*, 2007) or fluxomics (Sauer, 2006) have not been applied to mycobacteria. While metabolomics aims at measuring the concentration of as many as possible intracellular metabolites in a quantitative manner, fluxomics (or metabolic flux analysis) represents an approach that aimsat determining the rates of the intracellular metabolic reactions (or in other words the metabolic fluxes).

12.2. Towards obtaining a system understanding through mathematical modeling

Through systems biology omics analyses, as well as the traditional approaches to biology, molecular components and the interactions between these components can be identified. In order to obtain an understanding about how these interactions actually result in system behaviour, i.e. how a certain environment makes a cell express a certain phenotype, the mere knowledge about biological components and their interactions has to be quantitatively integrated into mathematical models and analyzed by computational means (Kitano 2002; Stelling 2004). This approach is typically referred to as the bottom-up branch of systems biology (Bruggeman and Westerhoff, 2007). For *M. tuberculosis*, two different modelling approaches have been applied to study bacterial metabolism and physiology:

12.2.1. Constraint-based models—The first modeling approach is called constraint-based modeling, a valuable tool for studying genome-wide metabolism (Feist and Palsson, 2008; Price *et al.*, 2003; Price *et al.*, 2004). In this approach, a mathematical version of an organism-specific metabolic network chart is computationally interrogated and analyzed. With this type of analysis, questions can be answered as to how carbon flow is redirected or how redox and energy cofactors are balanced upon knocking out a specific metabolic gene (Price *et al.*, 2003). In principle, this is what experienced physiologists do when they ‘manually’ inspect metabolic network charts. By means of computation, constraint-based modeling allows metabolic flux balancing on a genome-wide scale. With simple model

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organisms such as *E. coli* or *S. cerevisiae*, and also to a limited extend with mycobacteria, such analyses have proven to be useful in hypothesis generation and correction of errors in genome annotation and to some extent in predicting phenotypic behaviour (Duarte *et al.*, 2004; Feist *et al.*, 2007).

The most important input for this type of analysis is a mathematical model of the stoichiometry of an organism's metabolic network. For *M. tuberculosis*, three such models have been developed (Table 7). Raman *et al.* generated a stoichiometric model for the biosynthesis pathways of mycolic acid including 219 metabolic reactions (Raman *et al.*, 2005). The two stoichiometric models reconstructed by Beste *et al.* (2007a) and Jamshidi and Palsson (2007) have genome -wide scope and provide a comprehensive view on the mycobacterial metabolic network. The network reconstructed by Beste *et al.* (2007a) was calibrated against experimentally determined mycobacterial physiology and other experimental data and thus can be considered a validated model.

As a first step in the development of such a stoichiometric network model, a comprehensive list of the organism's metabolic reactions needs to be assembled. In this context, the genome annotation is usually a rich source of information, but also information from biochemistry and physiology is typically used to generate such a list (Joyce and Palsson, 2007). In a next step, the reaction list is then used to formulate mass balance equations for each metabolite present in the metabolic network. Applying the steady-state assumption to the balance equations, the set of differential equations translates into a set of linear equations that mathematically describes the metabolic reaction network at steady-state. Solely based on the genome annotation, typically the networks assembled are not complete, and gaps in essential biosynthesis pathways exist. In this context, information from the biochemical literature is exploited to close these gaps in the network. Additional further manual work and curation is necessary in order to define an equation that specifies the stoichiometry of a typical composition of the organism's biomass (as, for example, done by Beste *et al.*, 2005, for mycobacteria) and also typical directionalities of the metabolic reactions need to be defined (Kümmel *et al.*, 2006b). In addition to the reaction network described in such stoichiometric network reconstructions, information is typically also provided about the proteins and genes that are linked to metabolic reactions. Such, metabolic reconstructions represent a comprehensive and manually curated resource for an organism's metabolism.

The metabolic network model can also be interrogated using a great variety of computational methods. One of the most important tools for this is flux balance analysis (Kauffman *et al.*, 2003). Flux balance analysis uses mathematical optimization of a certain biological objective (i.e. typically maximization of biomass production per molecule of substrate) to determine values for each of the metabolic fluxes (i.e. reaction rates) in the network, which are within the constraints as defined by the stoichiometry of the metabolic network and the specified reaction directions. For this, Beste and colleagues have developed an internet-based tool to perform such calculations in a way that special mathematical expertise is not required besides a basic understanding of the concept behind this approach (Beste *et al.*, 2007a).

By applying the concept of flux balance analysis, *in silico* gene deletion studies were performed with all three presented model reconstructions for mycobacteria. With such computational analyses, the effect of gene deletion (i.e. removal of the corresponding metabolic reaction from the network) on an organism's viability under a given condition can be tested. Compared with the experimental global mutagenesis dataset on gene essentiality (Sassetti *et al.*, 2003), the *M. bovis* BCG model from Beste *et al.* (2007a) showed the best accuracy (78%) in correctly predicting growth phenotype.

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A disagreement found between the experimental and computational analysis points to relevant science. For example, an incorrect *in silico* lethality prediction points to incomplete knowledge about the metabolic network. As suggested from the analysis of Jamshidi and Palsson on *M. tuberculosis*(*in silico*), alternative pathways or enzymes have to exist for amino acid metabolism (Jamshidi and Palsson, 2007). The same seems to hold true for both fatty acid metabolism and membrane and peptidoglycan biosynthesis (Jamshidi and Palsson, 2007). These findings might also be due to unknown, and thus not considered, changes in the biomass composition, which is a required input for the *in silico* analysis.

The capability for efficient computational testing of gene deletion or inactivation effects (single or multiple), can be used as a fast and simple screen for putative targets for anti-tubercular drugs in metabolism. For example, the computational analysis of Beste *et al.* (2007a) revealed a potential role of the enzyme isocitratelyase during slow growth of mycobacteria, and experimentally it was indeed found that this enzyme shows higher activities in slow growing cells. Isocitratelyase has been shown to be essential for *M. tuberculosis* persistence in macrophages and mice (McKinney *et al.*, 2000).

Beyond the illustrated type of analyses mentioned, reconstructed stoichiometric networks models can also be used as an input for ^{13}C metabolic flux analysis, a technique that allows for the experimental determination of the magnitude of metabolic fluxes (Sauer, 2006). Furthermore, stoichiometric networks can be used for analyzing transcriptome or metabolome data. The metabolic network can link measured entities (i.e. transcripts or metabolites) and provide a context for the measured quantities, allowing for an improved statistical or mechanistic-based analysis (Cakir *et al.*, 2006; Kümmel *et al.*, 2006a; Patil and Nielsen, 2005; Zamboni *et al.*, 2008). Overall, genome-scale stoichiometric network models have been shown to be powerful tools for studying an organism's metabolism and physiology as they allow for the interrogation of the highly interconnected network of many metabolic reactions in a systematic and unambiguous fashion, which it is not possible with a mere inspection of the reaction map alone.

12.2.2. Detailed mechanistic models—A second modeling approach has recently been applied to *M. tuberculosis* where molecular interactions are described in detail with ordinary differential equations (Singh and Ghosh, 2006). This approach allows for dynamic and quantitative simulations of biological processes. Singh and Ghosh (2006) report some initial efforts to mathematically describe the kinetics of the tricarboxylic acid cycle and glyoxylate bypass in *M. tuberculosis*. Based on the developed mathematical model, these authors suggested the isocitrate lyase as a potential drug target, which agrees well with experimental data (Munoz-Elias and McKinney, 2005).

12.3. Outlook and challenges for systems biology with *M. tuberculosis*

Systems biology as a new biological discipline not only provides novel large-scale measurement techniques, but ultimately aims to achieve quantitative understanding of biological systems. Due to the complexity of biological systems, such understanding can most likely only be achieved by using mathematical models that can quantitatively and dynamically describe molecular interactions. In the mycobacteria field, only recent first efforts along these lines have been made.

It should be noted that mathematical models, by means of computational simulations, can generate novel hypotheses about the modeled biological system. These hypotheses can then be experimentally tested. Unfortunately, in the papers outlined above very little experimental follow-up has been performed to test the derived hypotheses. In order to realize the full potential of systems biology for mycobacterial research, two communities, i.e. those with the computational expertise and mycobacterial biologists, will need to work

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together to harvest the potential inherent to the systems biology modeling and computational analyses.

13. CONCLUSIONS

M. tuberculosis was discovered in 1882 by Robert Koch and it is estimated that over two billion people are infected with this bacterium. This is a remarkable feat when one considers that there is no significant animal or environmental reservoir and the limited genetic diversity exhibited by strains of *M. tuberculosis*. The bacterium makes up for these apparent weaknesses through its ability to evade the host immune defences and an extraordinary capacity to adapt to and survive under adverse conditions, including nutrient deprivation, hypoxia and various exogenous stress conditions. *M. tuberculosis* is also able to adapt to very different intracellular environments including: phagosomes in macrophages and dendritic cells (Russell, 2003), granulomas (Ulrichs and Kaufmann, 2006) and even fat cells (Neyrolles *et al.*, 2006). A key requirement for this adaptation is sensory perception and *M. tuberculosis* contains an array of signal transduction systems involving two -component regulatory systems, STPKs and ECF sigma factors. The ability of *M. tuberculosis* to efficiently accumulate nutrients in these different envir onments is essential for its survival, but many of the proteins involved in this process have not been determined. Uptake of these nutrients is tightly coupled to metabolism and energy generation and metabolism in the absence of cell replication is a hallmark of mycobacterial persistence. The electron donors and acceptors utilized under these conditions is an important question for mycobacterial energetics during persistence.

The search for new drug targets to combat the threat of *M. tuberculosis* is an area of renewed interest, especially drugs that will allow a shorter therapy. The contents of this review reinforce the idea that bacterial metabolism and energetics represents a virtually untapped source of new targets for anti-TB drugs. The hot prospects are those compounds that the target the F_1F_o-ATP synthase (Andries *et al.*, 2005), electron transport chain (Rao *et al.*, 2008; Weinstein *et al.*, 2005) and outer membrane proteins (Niederweis, 2008b). Outer membrane proteins offer the advantage as drug targets because potential inhibitors may not need to cross the outer membrane, which is an extremely efficient permeability barrier in mycobacteria. In addition, they are likely to represent novel drug targets because they do not appear to show any similarity to other proteins (Faller *et al.*, 2004).

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APPENDIX A

When cells are growing ideally (all cells have the same generation time) the age distribution is given by equation (1A) where a is a cell's age ($a = t/t_D$; $a = 0$ for new born cells and $a = 1$ for cells about to divide), and $t \leq t_D$ is the time (h) since the birth of a cell; n_c, is the number of cells per ml of culture;; $n_{c(a)}$, is the number of cells aged a per ml of culture; $n_{c(a=0)}$, is the number of new born cells per ml of culture.

$$n_{c(a)} = n_{c(a=0)} + e^{-a \bullet ; n2} \quad (1A)$$

The total number n_c of cells is given by equation (2A).

$$n_{c(a)} = n_{c(a=0)} \, {}_{a=0}\int^{a=0} e^{-a \bullet ; n2} \mathrm{da} \quad (2A)$$

Evaluation of equation (2A) leads to equation (3A).

$$n_c = 0.72 \, n_{c(a=0)} \quad (3A)$$

The age $a_{r(ORFi)}$at which ORF $_{(i)}$is replicated is given by equation (4A), as shown in the Theoretical section, where t_{G1} is the period between cell division and the onset of DNA replication, τ_g(h) is the period of DNA replication, $\gamma_{ORF(i)}$ is the replication coefficient for ORF$_{(i)}$and t_D is the doubling or generation time.

$$a_{r(ORFi)} = (t_{G1} + \gamma_{ORF(i)} \bullet \tau_g)/t_D \quad (4A)$$

Compared with the time taken to replicate the genome the time taken to replicate ORF$_{(i)}$ is very small and can be neglected. Suppose that the is a single copy of ORF $_{(i)}$, per genome, ($n_{ORF(i)/g=1}$). The number of copies of ORF$_{(i)}$per cell will depend on the cell's age. Before ORF$_{(i)}$is replicated (cells younger than $a_{r(ORFi)}$) the number of copies of ORF$_{(i)}$per cell is equal to $n_{ORF(i)/g}$; or a single copy per cell. For cells aged $a_{r(ORFi)}$and older the number of copies of ORF$_{(i)}$per cell will be equal to 2 $n_{ORF(i)/g}$. The number $n_{ORF(i)/c}$of copies of ORF $_{(i)}$per population -average cell is equal to the total number of copies of ORF$_{(i)}$divided by the total number of cells. Thus, $n_{ORF(i)/c}$ is defined in equation (5A).

$$n_{ORF(i)/c} = (n_{ORF(i)g}/n_c)\{n_{c(a=0)} \, {}_0\int^{ar(ORFi)} e^{-a \bullet \ln 2} \bullet \mathrm{da} + 2 n_{c(a=0)} \, {}_{ar(ORFi)}\int^{a=1} e^{-a \bullet \ln 2} \bullet \mathrm{da}\} \quad (5A)$$

The equation can be simplified by substituting for $n_c = 0.72 \, n_{c(a=0)}$. The integrated form of equation (5A) is presented in equation (6A)

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$$n_{\mathrm{ORF}(i)/c}=(n_{\mathrm{ORF}(i)/g}/(0.72\ln 2))\{[e^{-a\bullet\ln 2}]_{a}^{\mathrm{ar(ORFi)}}+2[e^{-a\bullet\ln 2}]_{\mathrm{ar(ORFi)}}^{1}\} \quad (6A)$$

An approximate equation related to equation (6A) was derived for *Escherichia coli* before genomic sequences were available (Bremer and Churchward, 1977; Chandler and Pritchard, 1975).

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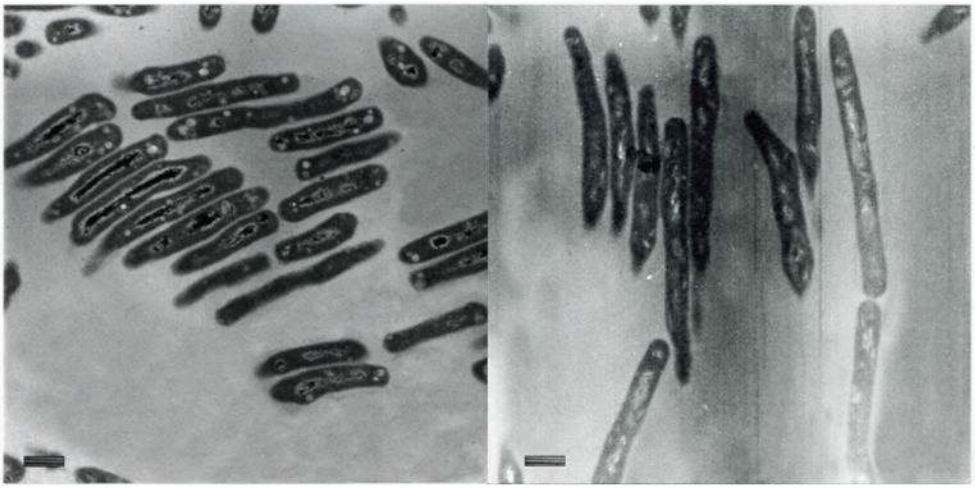

Figure 1. Electron micrograph of *M. smegmatis* mc^2 155

Cells were grown at 37°C with shaking (200 rev/min) in Kohn-Harris succinate medium plus 1% Tween 80. Cells were grown without (left panel, exponential phase) or with (right panel, late exponential phase/early stationary) a spring (10 cm long x 1.3 cm diameter) to increase agitation; in each case $\mu = 0.24\ h^{-1}$. For electron microscopy (Jeol JEM-100 Transmission Electron Microscope) cells were first fixed with 2.5% glutaraldehyde and sections were prepared. In each panel the bar represents 0.5 μm (R. Zaragoza-Centreras, R. A. Cox and J. A. Gonzalez-y-Merchand, unpublished work).

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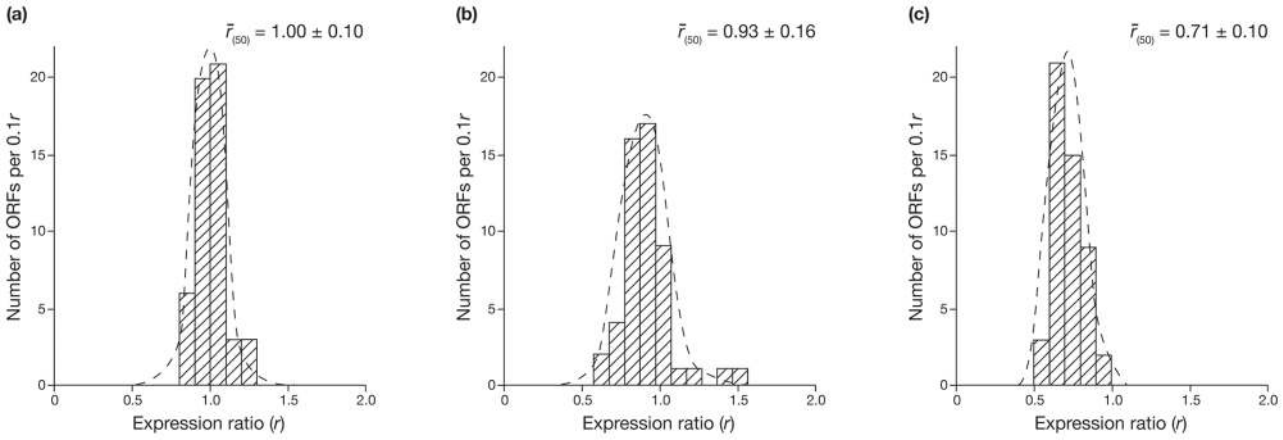

Figure 2. Comparison of the expression of r-protein genes of *M. tuberculosis* and *M. bovis* BCG grown under different conditions

In each panel the broken line denotes the values expected for a normal distribution according to the mean value and standard deviation cited.

(a) comparison of mid-exponential phase cells (optical density 0.15) when samples of the same cDNA preparation were labelled with fluorofors f' and *f&* Prime; (Voskuil *et al.*, 2004); Expression ratios were obtained from Voskuil *et al.* (2004) for a culture of *M. tuberculosis* clinical isolate 1254 grown at 37°C in 7H9 medium (supplemented with bovine serum albumin, NaCl, glucose and glycerol) shaking at 90 rpm. The expression ratio at this time point was obtained by using the RNA fraction to prepare one sample of cDNA labelled with fluorofor f' and another labelled with fluorofor f''. (b) comparison of *dosR* minus mutant with wild type *M. tuberculosis*(Kendall *et al.*, 2004); both wild type and mutant were found to grow at the same rate. The profile was computed from supplementary data.

(c) comparison of ribosomal gene expression for *M. bovis* BCG grown in a chemostat at two different specific growth rates; namely, 0.01 h^{-1}(f'') and 0.03 h^{-1}(f'); computed from the supplementary data of Beste *et al.* (2007b).

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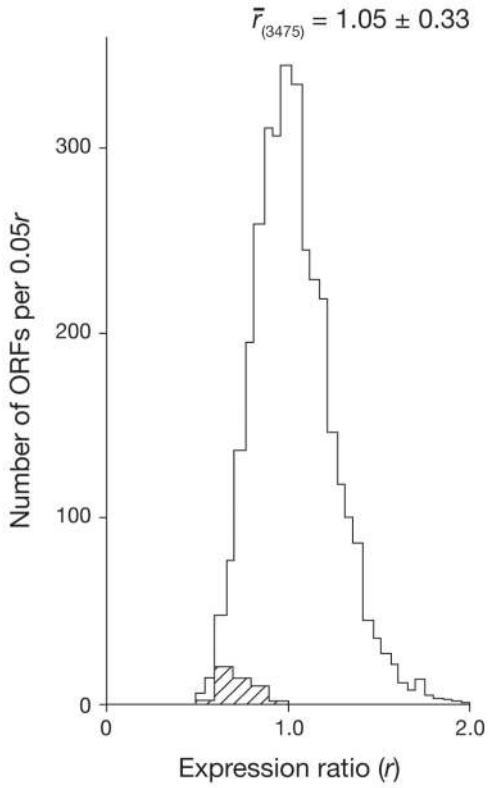

Figure 3. Comparison of the effects of slow growth on gene expression of *M. bovis* BCG: comparison of the expression ratios of all genes with r-protein genes

M. bovis BCG was grown in continuous culture at growth rates of 0.01 h^{-1} (r'') and 0.03 h^{-1}(r'), see Beste *et al.*(2007b). Supplementary data were used to calculate the distribution values of all 3475 ORFs investigated. The hatched section indicates the profile of r-protein genes.

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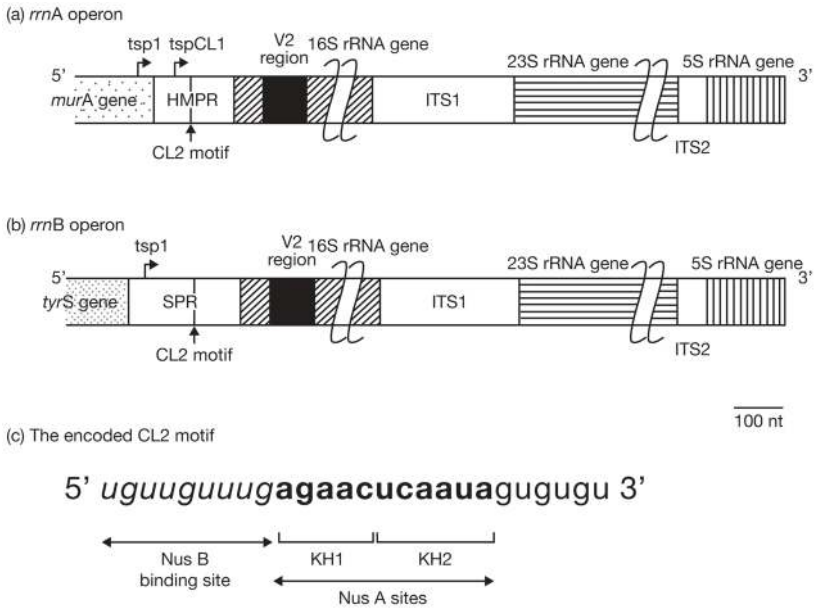

Figure 4. Organization of the *rrnA* and *rrnB* operons of mycobacteria

The operons are defined by the identities of their upstream genes. (a) *tsp*, transcription starting point; CL1, Conserved Leader sequence 1; HMPR, Hypervariable Mature Promoter Region. The number of promoters in this region ranges from 1 –4, each promoter is separated by 80 –100 base pairs; diagonal hatching, 16S rRNA coding region; ITS1, Internal Transcribed Spacer region 1; V2 region (black shading), variable region of 16S rDNA which serves to identify the species; horizontal hatching, 23S rRNA coding region; ITS2, Internal Transcribed Spacer region 2; vertical hatching, 5S rRNA gene.

(b) SPR, Single Promoter Region. All other abbreviations and shadings are defined in (a).

(c) Nucleotide sequence of the conserved CL2 motif and its NusA and NusB binding sites. The locations of this motif are shown in (a) and (b). Two regions of NusA (KH1 and KH2) are involved in binding to sequence shown in bold (Beuth *et al.*, 2005). The NusB component of the NusB. NusE (S10) dimer is thought to bind to the BoxA motif shown in italics (Das *et al.*, 2008).

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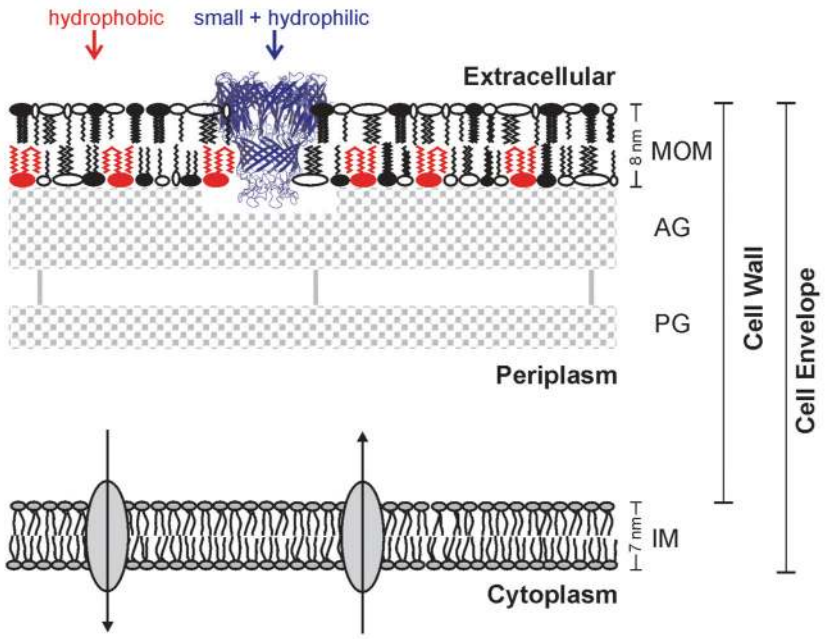

Figure 5. Transport processes across the mycobacterial cell envelope

Schematic representation of the mycobacterial cell envelope consisting of the inner membrane (IM) and the cell wall. This representation is based on cryo electron micrographs (Hoffmann *et al.*, 2007; Hoffmann *et al.*, 2008). Mycolic acids are covalently linked to the arabinogalactan (AG) – peptidoglycan (PG) copolymer and are an essential component of the inner leaflet of the outer membrane (OM). Extractable lipids are represented in black. The porin MspA mediates the uptake of small and hydrophilic nutrients such as sugars (Stephan *et al.,* 2005) and phosphates (Wolschendorf *et al.,* 2007) across the outer membrane of *M. smegmatis*. The MspA channel is 9.6 nm long (Faller *et al.,* 2004). Approximately 7 nm of the MspA surface are inaccessible to hydrophilic reagents (Mahfoud *et al.*, 2006). Hydrophobic compounds are assumed to diffuse directly across the outer membrane. The dimensions are approximately to scale.

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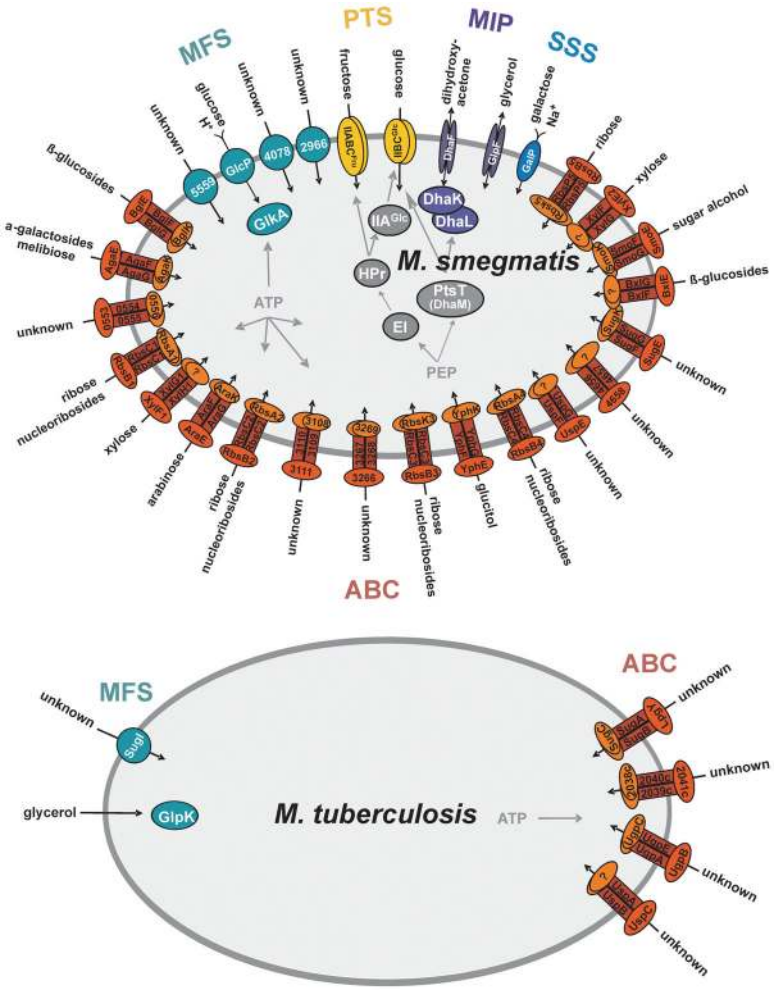

Figure 6. Transporters for uptake of carbohydrates across the inner membrane of *M.smegmatis* and *M. tuberculosis.*

Shown are the transport proteins of the ATP-binding cassette (ABC, red), phosphotransferase system (PTS, yellow), major facilitator super family (MFS, green), major intrinsic protein family (MIP, dark blue) and the sodium solute super family (SSS, blue). The derived putative substrates are inferred from *in silico* analyses in combination with experimental data (Titgemeyer *et al.*, 2007). Systems are denoted by the protein name instead of their locus tags (MSMEG_XXXX) if the substrates were experimentally verified or were predicted with a high likelihood. Note that the outer membranes of *M.smegmatis* and *M.tuberculosis* were omitted for clarity reasons. The figure was taken from Niederweis (2008b) with permission.

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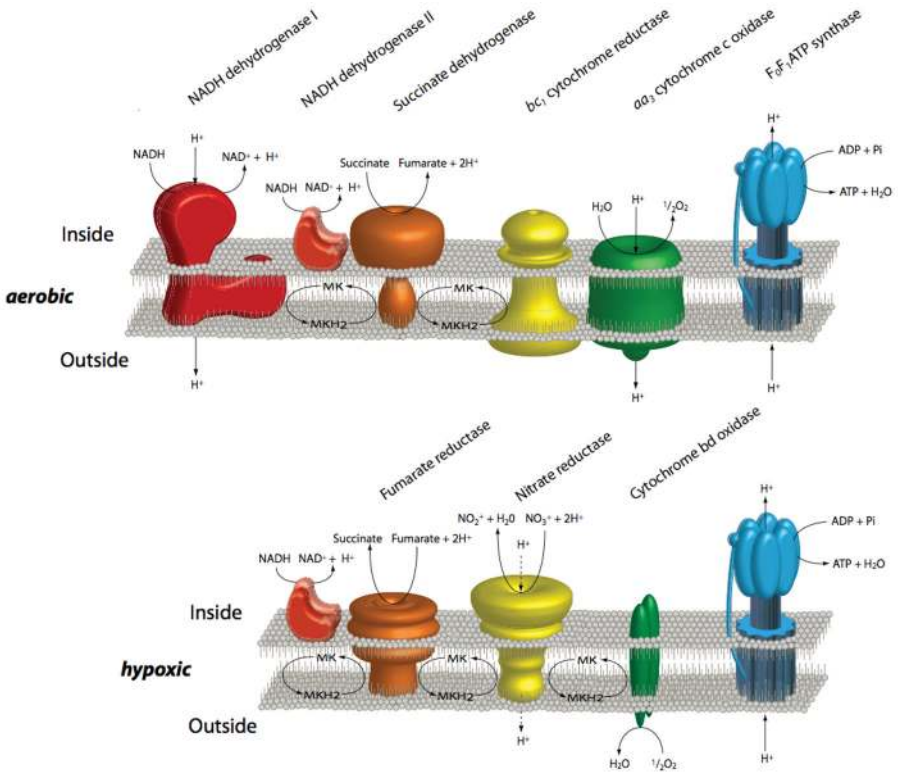

Figure 7. Schematic diagram outlining the electron transport chain components and ATP synthase of *Mycobacterium tuberculosis* growing under aerobic (top panel) and hypoxic conditions (bottom panel)

MK = menaquinone, MKH_2= menaquinol.

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Table 1

Features of Mycobacterium, Nocardia, Rhodococcus and Corynebacterium

Feature	*Mycobacterium*	*Nocardia*	*Rhodococcus*	*Corynebacterium*
% G+C	62 – 70	64 – 72	63 – 72	51 – 63
Cell shape	Mainly rods, sometimes branching	Mycelium fragmenting into rods and cocci	Scanty mycelium fragmenting into rods and cocci	Pleomorphic rods
Cell envelope	Waxy coat	Waxy coat	Waxy coat	Waxy coat
Mycolic acids	60 – 90 carbon atoms	46 – 60 carbon atoms	32 – 66 carbon atoms	22 – 66 carbon atoms
Growth rate (time for visible colonies)	2 – 40 days	1 – 5 days	1 – 3 days	1 – 2 days

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Table 2

DNA, RNA and protein contents reported for *M. bovis* BCG and *M. tuberculosis* H37Ra.

Property	*M. tuberculosis* H37Rv*	*M. bovis* BCG† (Glaxo)	*M. bovis* BCG‡	
Generation time (h)	2.40	2.40	23.10	69.30
DNA (fg/cell)	6.90§	6.90§	6.73¶	5.35¶
RNA (fg/cell)	232	13.3	9.96	2.24
Protein (fg/cell)	294	156.00	72.10	37.70

*Youmans and Youmans (1968). Cells were grown as a pellicle.

†Winder and Rooney (1970). Cells were grown in liquid culture.

‡Beste *et al.* (2005). Cells were grown in a chemostat.

§Values DNA, RNA and protein were calculated on the basis of one genome is equal to 4.79 fg and 1.45 genome per population.

¶Values DNA, RNA and protein were calculated on the basis of 1.40 and 1.10 genomes per population-average cell respectively for faster and slower growth rates.

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Table 3

Comparisons of observed and Eco_model derived specific growth rates of mycobacteria and other strains.

Species	Specific growth rate (h^{-1})		Eco_index
	Observed (μ)	Eco_model‡	
E. coli B/r	0.42[(a)]	0.42	1.00
M. tuberculosis H37Rv	0.043[(b)]	0.43	0.10
M. marinum strain M	0.17†[(c)]	0.32	0.53†
M. smegmatis mc^2155	0.23[(d)]	0.31	0.74
C. glutamicum ATCC13032	0.40†[(e)]	0.52	0.77†
S. coelicolor A3(2)	0.30†[(f)]	0.26	1.15†

† the subject strain was grown at 30°C.

‡ the Eco_model is defined in the text (see equation 1).

[(a)] Bremer and Dennis (1996);

[(b)] Wayne (1994);

[(c)] Clark and Shepard (1963);

[(d)] Sander *et al.* (1996);

[(e)] Frunzke *et al.* (2008);

[(f)] Shahab *et al.* (1996). See also Cox (2004)

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Table 4

Replication of *rrn* operons of *M. smegmatis* mc^{2-} 155.

Property	Strain		
	wild type	***rrnA* deletion**	***rrnB* deletion**
Generation time (h)	3	3	3
Duration of S-phase (h)	1.75	(1.75)	(1.75)
Duration of G1-phase (h)	0.67	(0.67)	(0.67)
Functional *rrn* operons	*rrnA*, *rrnB*	*rrnB*	*rrnA*
Cell age when replicated	0.56, 0.70	0.70	0.56
Number of copies/population-average cell	2.54	1.18	1.36

$ORF_{(0)}$(γ_{rep}= 0), The number of copies of $ORF_{(i)}$ range from 1.14 copies of $ORF_{(0)}$to 1.70 copies of $ORF_{(t)}$.

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Table 5

Ribosomal proteins S14, S18, L28 and L33 of *M. tuberculosis* H37Rv are each encoded by two genes.

Gene tag	Gene locus	*E.coli* equivalent	Essential gene	Size of protein (amino acids)	% similarities of orthologous proteins
Rv0717	*rpsN1*	S14		61	36
Rv2056c	*rpsN2*	S14	No	101	36
Rv0055	*rpsR1*	S18		84	54
Rv2055c	*rpsR2*	S18	No	88	54
Rv0105c	*rpmB1*	L28	No	94	53
Rv2058c	*rpmB2*	L28	No	78	53
Rv2057c	*rpmG1*	L33	No	54	30
Rv0634B	*rpmG2*	L33		55	30

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Table 6

Changes in the expression ratios of the genes encoding S14 with the period of cell culture of *M. tuberculosis*[#].

Gene tag	Encoded protein	Expression ratio		
		Period (days) of cell culture		
		0	8	14
Rv0717	*rps*N1 (S14) [*]	1.02	0.65	0.28
Rv2056C	*rps*N2 (S14)	1.15	7.8	10.4

[#] calculated from supplementary data of Voskuil *et al.* (2004).

[*] the expression ratios observed are typical of the majority of r proteins.

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Table 7

Overview of reconstructed stoichiometric network models of mycobacteria.

Intra-system Reactions/proteins	Metabolites	Genes	Reference
219/28	197	25	Raman *et al.* (2005)
849	739	726	Beste *et al.* (2007a)
939/543	828	661	Jamshidi and Palsson (2007)