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From Basic Science
to Patient Care



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Chapter 3: The Basics of Clinical Bacteriology

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3.1. The tubercle bacillus: a continuous taxon

Bacteria of the genus *Mycobacterium* are non-motile and non-sporulated rods. They are grouped in the suprageneric rank of actinomycetes that, unusually, have a high content (61-71 %) of guanine plus cytosine (G+C) in the genomic desoxyribonucleic acid (DNA), and a high lipid content in the wall, probably the highest among all bacteria. *Mycobacterium* and other closely related genera (i.e. *Corynebacterium*, *Gordona*, *Tsukamurella*, *Nocardia*, *Rhodococcus* and *Dietzia*) have similar cell wall compounds and structure, and hence show some phenotypic resemblance. Several mycolic acids in the envelope structure distinguish the mycobacteria. These quirky lipids may act as carbon and energy reserves. They are also involved in the structure and function of membranes and membranous organelles within the cell. Lipids constitute more than half of the dry weight of the mycobacteria. However, the lipid composition of the tubercle bacillus may vary during the life cycle in culture, depending on the availability of nutrients. The waxy coat confers the idiosyncratic characteristics of the genus: acid fastness, extreme hydrophobicity, resistance to injury, including that of many antibiotics, and distinctive immunological properties. It probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients.

Even exhibiting this common badge, the species within the genus *Mycobacterium* show great diversity in many aspects. Most of them live and replicate freely in natural ecosystems and seldom, if ever, cause disease. Only a few mycobacteria became successful pathogens of higher vertebrates, preferentially inhabiting the intracellular environment of mononuclear phagocytes. The host-dependent mycobacteria that cannot replicate in the environment are *Mycobacterium leprae*, *Mycobacterium lepraemurium*, *Mycobacterium avium* subsp. *Paratuberculosis*, and the members of the *Mycobacterium tuberculosis* complex. Bacteria within the *M. tuberculosis* complex are able to reproduce *in vitro*, in contrast to *M. leprae* and *M. lepraemurium*, which are uncultivable and require the intracellular milieu for survival and propagation.

Comprised within the *M. tuberculosis* complex and generically called the tubercle bacillus, the various etiologic agents of tuberculosis (TB) have distinct hosts, zoonotic potential and reservoirs. *M. tuberculosis*, and the regional variants or subtypes *Mycobacterium africanum* and “*Mycobacterium canettii*” are primarily pathogenic

in humans. *Mycobacterium bovis* and *Mycobacterium microti* are the causative agents of TB in animals, and can be transmitted to humans. Some particular strains isolated from goats and seals have been named *Mycobacterium caprae* and *Mycobacterium pinnipedi*, although sometimes they are identified as *M. bovis* subspecies or variants. It could be expected that the major evolutive shifts involved in adaptation to different hosts would have entailed significant microbiological differentiation. However, the above mentioned agents of TB together with the vaccine bacille Calmette-Guérin (BCG) strains rank close to each other along a phenotypically continuous taxon (David 1978, Wayne 1982, Vincent 1992, van Soolingen 1997, van Soolingen 1998, Niemann 2000, Niemann 2002, Sola 2003, Mostowy 2005). Phenotypic differentiation is consistently clear-cut between the extreme species within the taxon, i.e. *M. tuberculosis* and *M. bovis*, but differences between species comprised within these two extremes are much less defined. The close affiliation among the members of the complex is endorsed by high genomic DNA similarity. At the same time, some molecular markers allow species differentiation within the complex (see chapter 2).

Table 3-1: Lineage of the agents of TB.

<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Tree&id=1760&lvl=3&lin=f&keep=1&srchmode=1&unlock>

Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	<i>Mycobacterium</i> unique genus
Species	<i>M. tuberculosis</i> <i>M. bovis</i> <i>M. africanum</i> <i>M. microti</i> "M. canettii" <i>M. caprae</i> <i>M. pinnipedi</i>

In general, systematic and clinical mycobacteriologists accept new taxa at a slow pace. This is why the taxonomic status of some new members of the complex is still uncertain (see LPSN, <http://www.bacterio.cict.fr> and DSMZ, http://www.dsmz.de/microorganisms/bacterial_nomenclature.php). At the same time, the rank and species assignment have been questioned in other cases (Niemann 2003, Niemann 2004). The value of phenotypic and genotypic traits in the definition of a species in the complex should be reconsidered to meet new widely accepted definitions.

3.2. Microscopic morphology

The microscopic appearance does not allow the differentiation of the pathogenic agents of TB, mainly *M. tuberculosis*, from other mycobacteria although some characteristics may be indicative. In smears stained with carbol fuchsin or auramine and examined under light microscope, the tubercle bacilli typically appear as straight or slightly curved rods. According to growth conditions and age of the culture, bacilli may vary in size and shape from short coccobacilli to long rods. A typical curved shape has been described for *M. microti* (van Soolingen 1998). The dimensions of the bacilli have been reported to be 1-10 μm in length (usually 3-5 μm), and 0.2-0.6 μm in width. Therefore, the length of the microorganism is comparable to the diameter of the nucleus of a lymphocyte. Unlike some fast growing mycobacteria and other actinomycetales, *M. tuberculosis* is rarely pleomorphic, it does not elongate into filaments, and does not branch in chains when observed in clinical specimens or culture. In the experimental macrophage infection, intracellular bacilli were described as being significantly elongated compared to broth-grown bacilli and, remarkably, to display bud-like structures (Chauhan 2006).

When numerous and actively multiplying, the bacilli are strongly acid fast and show an evident and distinctive tendency to form hydrophobic bundles (Figure 3-1 and 3-2). Free bacilli can also be seen, though, especially at the border of the swarms. In unlysed host tissue, the bacilli are more numerous within the phagocytic cells.

Once the disease has been controlled, dying bacilli become sparser, often faintly and unevenly colored, due to partial loss of the internal contents. Of course, irregular staining may also be the consequence of technical defectiveness of dyes or staining procedures.



Figure 3-1: Ziehl-Neelsen staining of *Mycobacterium tuberculosis* growing in culture at 1000x magnification.

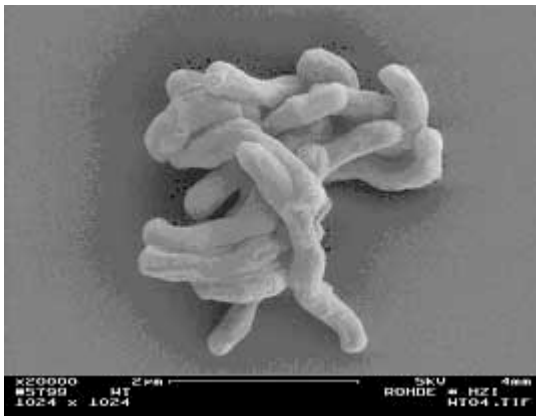


Figure 3-2: Electron microscopy of *Mycobacterium tuberculosis* growing in culture (Courtesy of M. Rohde -M. Singh).

The light microscope examination can not resolve the internal structures of the tubercle bacillus with the exception of some intracellular lipid vacuoles appearing as unstained spherules at regular intervals inside the bacilli (Draper 1982) and deposits of lipophilic material that might have a storage function (Garton 2002). Despite considerable efforts, a more subtle resolution of the ultrastructure of the bacillus has not been achieved. This is probably due to technical problems arising from biosafety, from the minute size of the bacilli, and from the large amounts of

complex lipids existing in their wall. With electron microscopy, some inner dense granules can be identified. They are believed to consist of polyphosphate and might be an energy store in the cell and also the site of oxidation-reduction reactions. In sections of the cell, the plasma membrane is seen to proliferate into vesicular or laminated internal bodies that might supply metabolic activities. Ribosomes, DNA filaments and radial bands, the latter postulated to be remaining scars of cell division, have also been described (Draper 1982, Brennan 1994).

Recently, the initiation of septum formation prior to division was clearly evidenced by tagging the mid-cell rings with green fluorescent protein (Chauhan 2006). Also, impressive images of the surface of *M. bovis* BGG were obtained by atomic force microscopy (Verbelen 2006).

3.3. Cell wall structure

As the most distinctive anatomical feature of the bacillus, the cell envelope has been the main object of research. Progressive chemical, molecular and ultrastructural research has produced robust knowledge on the synthetic pathways and structure of the mycobacterial cell envelope (Draper 1982, Brennan 1994, Draper 2005, Kremer 2005). The envelope, which has been profusely represented by schematic models, is composed of the **plasma membrane**, a **cell wall**, and an **outer capsule like layer**.

The **cytoplasmic membrane** of mycobacteria does not seem to be peculiar except for the presence of some lipopolysaccharides that are anyway shared by all actinomycetales (Mahapatra 2005). This vital interface provides osmotic protection, regulates the traffic of specific solutes between the cytoplasm and the environment, and subsumes the cell house-keeping tasks. The membrane contains proteins with different functions, i.e. sensors measuring the concentration of molecules in the environment, proteins translocating signals to genetic and metabolic machinery in the cytoplasm, enzymes involved in metabolic processes and energy generation, and carriers mediating selective passage of nutrients and ions. The enzymes intervene in cell wall and membrane synthesis, septum formation during cell division, assembly and secretion of extracytoplasmic proteins, and DNA replication. Still, very little is known specifically about the membrane of *M. tuberculosis*.

The membrane is surrounded, as in almost all bacteria, by a **cell wall** that protects the cell contents, provides mechanical support and is responsible for the characteristic shape of the bacterium. The mycobacterial cell wall, however, is unique among prokaryotes. The wall is constituted by an inner peptidoglycan layer, which

seems to be responsible for the shape-forming property and the structural integrity of the bacterium. The structure of this stratum differs slightly from that of common bacteria, as it presents some particular chemical residues and an unusual high number of cross-links. Indeed, the degree of peptidoglycan cross linking in the cell wall of *M. tuberculosis* is 70-80 % whereas that in *E. coli* is 20-30 %.

Covalently bound to the peptidoglycan is a branched polysaccharide, the arabinogalactan, whose outer ends are esterified with high molecular weight fatty acids called mycolic acids. These components are peculiar as the arabinogalactan has unusual components and linkages and the mycolic acids are typically long and branched chains containing 60- to 90-carbon atoms. The genera *Dietzia*, *Rhodococcus*, *Nocardia*, *Gordona*, and *Mycobacterium* have mycolic acids with increasing average numbers of carbon atoms. The arrangements of these mycolic acids are species-specific, a property that allows the identification of many species of mycobacteria by gas-liquid, high-performance liquid or thin-layer chromatography (see chapter 14). The mycolic acids specific to *M. tuberculosis* are alpha, keto and methoxymycolates containing 76 to 82, 84 to 89, and 83 to 90 carbons respectively.

The outer layer of the cell wall presents an array of free lipids such as phthiocerol dimycoserates (PDIM), phenolic glycolipids (PGL), trehalose-containing glycolipids and sulfolipids (SL). The unusual "*M. canettii*", with its smooth colony morphology, has a unique phenolic glycolipid (van Soolingen 1997). *M. bovis* and *M. bovis* BCG produce sizable amounts of a PGL designated as mycoside B, whereas most *M. tuberculosis* strains are deficient in this component.

Traversing the whole envelope, some glycolipids such as the phosphatidyl-myoinositol mannosides, lipomannan (LM) and lipoarabinomanan (LAM), are anchored to the plasma membrane and extend to the exterior of the cell wall. LAMs are species-specific. The mycobacterial wall also contains interspersed proteins. Some are in the process of being exported, some might be residents. Several of these proteins are responsible for cell wall construction during the life of the bacillus. There are also certain proteins called porins forming hydrophilic channels that permit the passive passage of aqueous solutes through the mycolic acid layer. Mycobacterial porins seem to be different from those of gram-negative bacteria.

While growing in a static liquid culture or within a human cell, *M. tuberculosis* seems to accumulate an unbound **pseudo-capsule**. Apparently, when the medium is disturbed, the capsule separates, leaving the lipophilic surface exposed. In fact, the capsule components have largely been recognized in culture filtrates but its structure and location were resolved rather recently. The capsule contains proteins, polysaccharides and minor amounts of inner lipids, which are apparently in con-

stant turnover. The constituents of the capsule might be shed *in vivo* within the infected host cells. It has been proposed that the capsule might be protective and bioactive. In addition, a number of envelope-associated substances have been described, mostly lipids and glycolipids.

The tubercle bacillus shares most ultrastructural features with other members of the genus, including non-pathogenic mycobacteria. Its distinctive ability to survive in mammalian hosts, its pathogenicity and its immunogenic properties seem to derive, at least in part, from the nature of some of the molecules of the bacterial wall (Riley 2006, Smith 2003).

The envelope of the tubercle bacillus seems to be a dynamic structure that can be remodeled as the microorganism is either growing or persisting in different environments (Kremer 2005). In fact, in growth conditions interfering with the synthesis of the wall, *M. tuberculosis* may be induced to produce wall-deficient spheroplasts that apparently are not pathogenic unless they revert to being normal bacteria (Ratnan 1976). Cell wall thickening was observed in oxygen-deficient conditions (Cunningham 1998). Besides, the expression of genes that putatively code for porins seems to be up regulated in certain environmental conditions, such as mildly acidified culture medium, as well as inside the macrophage vacuoles (Draper 2005).

3.3.1. Acid fastness

Unlike Gram-negative bacteria, mycobacteria do not have an additional membrane in the outer layers of the cell wall. They are structurally more closely related to Gram-positive bacteria. However, mycobacteria do not fit into the Gram-positive category as the molecules attached to the cell wall are distinctively lipids rather than proteins or polysaccharides. Frequently, they do not retain the crystal violet and appear as “ghosts” after Gram staining. The waxy cell wall of mycobacteria is impermeable to aniline and other commonly used dyes unless these are combined with phenol.

To discover the causative agent of TB, Robert Koch had to develop a specific staining process using alkaline dyes. Soon after, Ehrlich discovered the acid fastness of the tubercle bacillus, which has been the prominent characteristic of mycobacteria up until now. The expression “acid-fastness” describes the resistance of certain microorganisms to decolorization with acid-alcohol solutions after staining with arylmethane dyes such as carbol fuchsin. This feature is of utmost practical

importance in identifying the tubercle bacillus, particularly in pathological specimens.

In spite of being a hallmark, the wall permeability to alkaline dyes and the mechanisms preventing their removal by acids are still not totally understood in molecular terms. Most of the current knowledge on this phenomenon was disclosed in pioneer experiments. The beading observed inside the cells was interpreted as accumulation of free dye rather than staining of particular structures, which led to the early hypothesis that alkaline stains are retained in the cytoplasm (Yegian 1947). Later, evidence was provided sustaining the role of lipids in trapping the dyes. Indeed, there is a parallelism between the increasing degree of acid fastness displayed by microorganisms in the genera *Corynebacterium*, *Nocardia*, and *Mycobacterium*, and the increasing length of mycolic acid chains in their walls. This correspondence suggests that the chemical binding of the dye to these molecules might be a determinant for acid fastness.

Bacilli suspended in aqueous solution retain the acid fastness for a long time, even after heating. However, the property is absolutely dependent on the integrity of the bacillus. Unimpaired mycolic acids are required to hinder the penetration of water-soluble dyes and bleaching acids (Goren 1978). The acid fastness of the bacillus is obliterated by cell trauma or autolysis (Baisden 1942), infection by specific mycobacteriophages (Gangadharam 1976) or treatment with antibiotics targeting cell-wall synthesis, such as isoniazid (INH) (Mohamad 2004). Acid fastness seems to also be dependent on nutrients and oxygen tension, as suggested by fluctuations in staining observed in different culture conditions (Nyka 1971). Dormant *M. tuberculosis* bacteria bearing cell wall alterations may remain undetected by the classic Ziehl-Neelsen staining (Seiler 2003).

3.3.2. Cord formation

By microscopic observation, Robert Koch first described the arrangement of bacilli in braided bunches and associated this phenomenon with virulent strains of *M. tuberculosis*. He also detailed the aspect of cultures in blood serum as compact scales which could be easily detached. In general, fresh virulent *M. tuberculosis* bacilli produce rough textured colonies on solid media, expanded gummy veils on the surface of liquid media and serpentine on microscopic smears. In contrast, non-virulent mycobacteria and tubercle bacilli attenuated by prolonged cultures usually develop smooth colonies on solid media, form discrete mats in liquid media and distribute randomly in loose aggregates when smeared. The recognition of these two peculiarities, cording and crumbly colony formation, provides a reliable

and timely clue to the experienced microbiologist for the presumptive distinction of *M. tuberculosis* from other mycobacteria in cultured specimens and even in sputum smears (see chapter 12).

These distinctive characteristics of the virulent bacilli have been attributed to the trehalose 6, 6'-dimycolate. This compound, also known as cord factor, was described as an extractable glycolipid consisting of two mycolic acid molecules loosely bound in the outer layer of the cell wall (Noll 1956). A myriad of biological activities related to pathogenicity, toxicity, and protection against the host response have been attributed to this molecule. However, it does not seem to be essential for bacterial multiplication *in vitro* (Indrigo 2002).

Several models were used to identify the role of the trehalose 6, 6'-dimycolate (TDM) in the microscopic and macroscopic morphology of *M. tuberculosis*. In this way it was demonstrated that beads coated with this substance generate an oriented hydrophobic interaction and aggregate in elongated structures similar to cords (Behling 1993). Later, the molecular packing of TDM was imitated (Almond 1996). Recently, immunohistochemistry was used to investigate the distribution of TDM in *M. tuberculosis* culture pellicles. According to the results of this experiment it was proposed that the TDM released by the microorganism molds a rigid hydrophobic interphase that is responsible for the cultural and microscopic appearance of virulent bacilli (Hunter 2006).

However, this phenomenon is not yet clearly understood. One matter of confusion is the fact that TDM is also present in other non-cording avirulent mycobacteria. Taking this into consideration, the activity of the cord factor in *M. tuberculosis* has been ascribed to a particular surface conformation (Schabbing 1994) and to the large amounts of this molecule released by the tubercle bacilli (Hunter 2006). The localization of DNA sequences encoding cording has not yet been elucidated. Five genes probably associated with cord formation were identified, but their real implication has not been demonstrated (Gao 2004).

So far, the characteristics of the TDM of "*M. canettii*", a human pathogen that produces unusually smooth colonies, have not been described.

3.3.3. Permeability barriers

The tightly packed mycolic acids provide the bacillus with an efficient protection and an exceptional impermeability. In addition to the capsule, an even thicker layer of carbohydrate and protein outside the lipid layer impedes the diffusion of large molecules, such as enzymes, and protects the lipid layer itself. The shell restricts

the permeability to most lipophilic molecules. Other substances can bypass this barrier through the porins, although this mechanism is not very efficient: *M. tuberculosis* possesses a low number of porins compared to other bacteria and the porins admit only small water soluble molecules (Niederweis 2003).

Several experiments have been performed that have provided the rationale for the long believed concept that impermeability is at least one of the determinants for two *M. tuberculosis* characteristics: its slow growth and its intrinsic drug resistance. The penetration rate of β -lactam antibiotics into *M. tuberculosis* was found to be comparable to that of *Pseudomonas aeruginosa* and approximately 100 times lower than that of *Escherichia coli* (Chambers 1995). In recombination experiments, the expression of the *M. smegmatis* porin MspA was followed by increased susceptibility of the tubercle bacillus to β -lactam antibiotics and even to first-line anti-tuberculous drugs. At the same time, the expression of the same porin in *M. bovis* BCG stimulated the uptake of glucose and accelerated growth (Mailaender 2004).

Treatment with some drugs that are known to fray or somehow alter the surface architecture of the cells was shown to increase the susceptibility of *M. tuberculosis* (Verbelen 2006). In effect, at sub-inhibitory concentrations, ethambutol and dimethyl sulfoxide enhanced the activity of anti-tuberculosis drugs against *M. tuberculosis* strains that were originally resistant to these drugs (Jagannath 1995). Similarly, some antidepressants, such as chlorpromazine, have in vitro activity themselves against the tubercle bacillus (Ordway 2003).

3.4. Nutritional and environmental requirements for growth

The tubercle bacillus is prototrophic (i.e. it can build all its components from basic carbon and nitrogen sources) and heterotrophic (i.e. it uses already synthesized organic compounds as a source of carbon and energy). The microorganism macromolecular structure and physiological (metabolic) capabilities result in high adaptation to the specific environment. In turn, the nutritional quality of the environment determines the bacillus lifestyle and limitations, either in the natural habitat or in culture media, as do various physical conditions such as oxygen availability, temperature, pH and salinity.

As the environment changes, the bacillus is able to bring into play different physiological pathways in order to survive even in harsh conditions. This is a highly resourceful strategy, not only for pathogenicity but also for species persistence. It has been shown that, during the course of infection in mice, *M. tuberculo-*

sis metabolism may shift from an aerobic, carbohydrate-metabolizing mode to one that is more microaerophilic and utilizes lipids (Segal 1956). These demonstrations, which were reported a long time ago, were supported in recent times by the complete sequencing of the *M. tuberculosis* genome in which an unusually high number of genes putatively involved in fatty acid metabolism were identified. This phenomenon may be related to the ability of the pathogen to grow or persist in host tissues where fatty acids may be the major carbon source (Neyrolles 2006) (see chapter 4).

In vitro, the members of the *M. tuberculosis* complex are not fastidious unless damaged by some noxious agents. In fact, the medium used by Koch to cultivate *M. tuberculosis* was simply sterile coagulated blood serum. The tubercle bacilli can also grow in salt solutions using glycerol as a carbon source, ammonium ions and asparagine as nitrogen sources, and micronutrients. *M. tuberculosis* is able to metabolize glycerol into pyruvate, whereas *M. bovis* is not. Indeed, the genome sequence analysis confirmed that all the genes required for the formation of pyruvate are non-functional in *M. bovis*. Being defective in this metabolic process, *M. bovis* grows much better in the presence of a pyruvate salt as a source of carbon. Albumin, which is normally provided by adding eggs or bovine serum albumin to the culture media, promotes the growth of these microorganisms. Other subsidiary media components may be used, such as Tween 80, a detergent that disperses the bacilli in liquid media. It was postulated that bovine serum albumin may bind the excess of oleate that can be released from the detergent up to toxic amounts. Biotin and catalase have been incorporated to the Middlebrook series media to stimulate the revival of damaged bacilli in clinical specimens (Wayne 1982).

Trace elements found by the microorganism in the water, inorganic ions, small molecules, and macromolecules have either a structural or a functional role in the cell. Magnesium and iron are essential for life. A deficiency in these elements frequently reduces the virulence of bacterial pathogens, including the tubercle bacillus. As iron is usually in the form of insoluble ferric salts in the environment, special iron systems are required to incorporate this element into the cell. Exochelins and mycobactins are the major siderophores used by mycobacteria to perform this function. The former are hydrophilic peptides secreted into the environment for iron gathering. The latter are hydrophobic compounds located within the cell wall to introduce the iron into the cytoplasm. The *mbt* operon is putatively involved in the synthase activities required to produce the mycobactin core (De Voss 2000). The incorporation of mycobactin into culture media can promote the growth of ailing *M. tuberculosis* isolates.

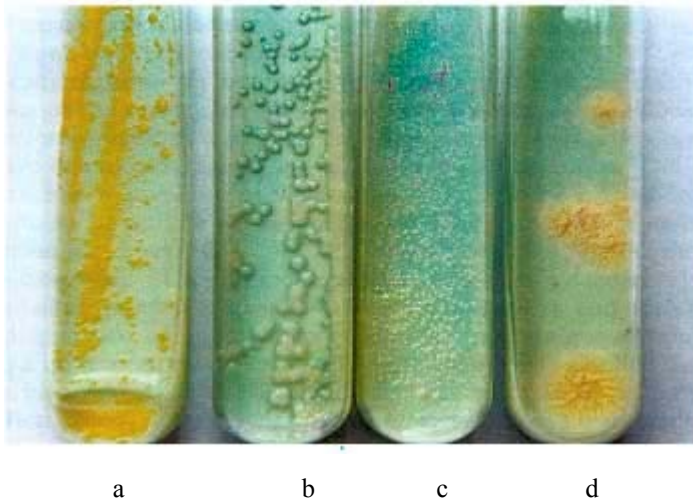


Figure 3-3: Mycobacteria growing on Löwenstein-Jensen slants. a. *Mycobacterium gordonae*; b. *Mycobacterium fortuitum*; c. *Mycobacterium avium*; d. *Mycobacterium tuberculosis*.

The tubercle bacillus requires oxygen as a final electron acceptor in aerobic respiration. Molecular oxygen is reduced to water in the last step of the electron transport system. In nature, the bacillus grows most successfully in tissues with high oxygen partial tension, such as the lungs, particularly the well-aerated upper lobes. Carbon dioxide is essential and may be taken from the atmosphere and also from carbonates or bicarbonates. In the laboratory, an atmosphere of 5 to 10 % carbon dioxide favors culture growth, at least during the early stage of incubation. On the other hand, *M. bovis* is microaerophilic, i.e. it grows preferentially at a reduced oxygen tension.

M. tuberculosis is mesophile and neutrophile as its multiplication is restricted to conditions offered by warm-blooded animals: about 37°C and a neutral pH. The temperature and hydrogen ion concentration ranges, in which the bacillus is able to multiply, are relatively narrow. High saline concentration such as that found in media containing 5 % sodium chloride, inhibits the growth of the microorganism.

3.5. Generation time

Under favorable laboratory conditions, *M. tuberculosis* divides every 12 to 24 hours. This pace is extremely slow compared to that of most cultivable bacteria, which duplicate at regular intervals ranging from about 15 minutes to one hour. Recently, the low multiplication rate of the tubercle bacillus was nicely exposed by Chauhan *et al.* These authors demonstrated the small proportion of cells initiating the septation process prior to division among tubercle bacilli growing either in broth or inside macrophages (Chauhan 2006).

The slow growth rate might be partially determined by the cell wall impermeability that limits nutrient uptake. However, only a minimal stimulus to bacterial multiplication is achieved when the permeability is increased through treatment with some compounds that interact with the cell envelope. Harshey and Ramakrishnan identified ribonucleic acid (RNA) synthesis to be a major factor associated with the long generation time of the tubercle bacillus. They demonstrated that both the ratio of RNA to DNA and the RNA chain elongation rate are ten-fold lower in *M. tuberculosis* compared to *E. coli* (Harshey 1977). Another unusual feature is the existence of a unique operon commanding RNA synthesis. Furthermore, when the tubercle bacillus switches from the stationary to the active multiplying phase, its total RNA content increases only twofold. Consequently, the protein synthesis must be retarded (Verma 1999). The influence of nutrient availability on the ribosome synthesis rate, which is a proxy of metabolic activity, remains controversial (Hampshire 2004).

The low multiplication rate explains the typically sub-acute to chronic evolution of the disease and the long time required to attain visible growth *in vitro*. Numerous experiences using different nutrients and culture conditions have demonstrated that some factors may abrogate a lag in adaptation of the bacilli in culture media but, once growth is initiated, the replication cycle will still take no less than 12 hours. This limitation in accelerating the tubercle bacillus growth could not be overcome. Instead, the main achievements for diagnosis have been made through the use of tools that enable the detection of a minimal quantity of bacilli in the media. First, transparent agar medium allowing the detection of tiny colonies were introduced; more recently, the addition of biosensors has been adopted to detect redox changes produced by the bacilli metabolism (see chapters 12 and 14).

3.6. Metabolic and biochemical markers

In the laboratory, the classical phenotypic identification, speciation and subspeciation of members of the *M. tuberculosis* complex include key diagnostic tests developed to detect certain metabolic intermediates and the activity of some enzymes that are essential for life and pathogenicity. In addition to some susceptibility tests, the investigation of niacin accumulation, nitrate reductase and urease activity allows the distinction of *M. tuberculosis* complex and species differentiation within the complex (see chapter 8). Most of the information on the structure and function of these metabolites and enzymes has focused on *M. tuberculosis* and, to some extent, on *M. bovis*. Much less is known about these features in other members of the *M. tuberculosis* complex.

Niacin (nicotinic acid) plays a vital role in organic life, as it is involved in the oxidation-reduction reactions of energy metabolism and in the DNA repair processes. Although all mycobacteria produce niacin, most of them employ the majority of the yielded metabolite in the synthesis of co-enzymes. In contrast, *M. tuberculosis* produces and accumulates substantial amounts of niacin as a result of a very active nicotinamide adenine dinucleotide degradation pathway and the inability to process the resultant niacin (Kasarov 1972). In vitro, *M. tuberculosis*, "*M. canettii*", and some isolates of *M. africanum* excrete water-soluble niacin into the culture media, the detection of which is extremely useful for definitive identification. This is another hallmark that has not been investigated in molecular terms. Again, most of the knowledge existing on this phenomenon and the tools for its detection were produced a long time ago by bacteriological and chemical studies.

Like many aerobes, including other mycobacteria, the tubercle bacillus depends upon certain enzymes to detoxify lethal oxygen radicals, such as peroxides and H_2O_2 , which are self-generated during respiration or produced by host phagocytes. The main *M. tuberculosis* antioxidant enzyme that can hydrolyze H_2O_2 is a heat-labile catalase-peroxidase with both catalase and peroxidase activities. The thermal lability of this enzyme is a marker of the *M. tuberculosis* complex. *M. tuberculosis* also has an alternative alkyl-hydroperoxidase, which is postulated to compensate for the lack of catalase activity. Paradoxically, the catalase is not only self-protective but can also be self-destructive as it activates the anti-tuberculous pro-drug INH. Mutations in the genes encoding both enzymes (*katG* and *ahpC*) are involved in resistance to INH and thus, have been the subject of active investigation (see chapter 18). Understandably, resistance to INH may be associated with irregular catalase activity. Among the biochemical markers commonly investigated for mycobacteria identification in the clinical microbiological laboratory, this is the only one that may be affected by drug resistance to some extent.

Even though *M. tuberculosis* prefers ammonium and asparagine, it can deficiently utilize nitrate and nitrite as sole sources of nitrogen *in vitro*. It has been speculated that, in infected hosts, the microorganism might use nitrate as a nitrogen source and/or as a terminal electron acceptor in the absence of oxygen. Whatever the physiological function may be, *M. tuberculosis* has an enzyme bound to the cell membrane that rapidly reduces nitrate and leads to the accumulation of nitrite. Unlike those of other mycobacteria, *M. tuberculosis* nitrate reductase is permanently very active *in vitro* regardless of the culture conditions. Under hypoxic conditions or on exposure to nitric oxide, its activity may even be enhanced by induction of the protein NarK2. This protein is a nitrate transporter that might be able to sense the redox state of the cell and adjust its own activity accordingly (Sohaskey 2005). The reductase activity may be hindered by very high concentrations of INH. Furthermore, some isolates of the tubercle bacillus that are resistant to INH and para-aminosalicylic acid (PAS) were found to be unable to reduce nitrate when growing in minimal media (Hedgecock, 1962). The nitrate reductase activity seems to be encoded by the constitutive *narGHJI* operon (Weber 2000), which is present in both *M. tuberculosis* and *M. bovis*. However, *M. bovis* does not reduce nitrate. It was demonstrated that a single nucleotide polymorphism at position 215 in the promoter of this gene cluster determines different levels of enzyme activity in both species (Sohaskey 2003). “*M. canettii*” and some isolates of *M. africanum* produce detectable amounts of nitrite from nitrate *in vitro*.

M. tuberculosis is able to produce ammonia from urea by a urease-mediated reaction. The ammonium can be then used by the microorganism for biosynthesis. The urease is coded by the genes *ureABC* (Reyrat 1995) and it might also be important for nitrogen acquisition as its activity increases when nitrogen sources are limited (Clemens 1995). In addition, the consequent alkalization of the microenvironment by ammonium ions might inhibit the maturation of phagolysosomes and contribute to the defective maturation of major histocompatibility complex class II molecules of host monocytes (Sendide 2004).

3.7. Resistance to physical and chemical challenges

Although the tubercle bacillus is not a spore-forming bacterium, it has a remarkable capacity to endure unfavorable conditions. The bacillus is able to circumvent destruction within the macrophages and to limit the access to the bacterial targets of hydrophilic antiseptics and antibiotics (see Chapters 5, 11, and 18). For example, chloride and bromide salts of cetylpyridium do not impair the viability of the tubercle bacilli for at least 14 days (Tazir 1979, Pardini 2005). Therefore, these salts are

used as preservatives when the processing of specimens is delayed. Likewise, the natural impermeability of the bacterium to common hydrophilic antimicrobial agents is used in the clinical mycobacteriology laboratory. In effect, some broad spectrum antibiotics are added to selective media to isolate the tubercle bacillus.

As already mentioned, *M. tuberculosis* complex organisms multiply within narrow temperature and pH ranges, and at a high oxygen tension, which is indicative of the effect produced by these physical conditions on the rates of enzymatic reactions. However, the tubercle bacilli can withstand conditions far distant from those optimal for propagation. The bacillus survives to some extent in the acid or alkaline microenvironment as a result of its interaction with the defensive mechanism of the host, as well as the acid contents of the stomach. Similarly, a significant proportion of the bacilli population present in clinical specimens can endure a brief treatment with diluted solutions of acids and alkalis such as sulfuric acid or sodium hydroxide. This property is peculiar as most microflora present in the specimens are killed by this treatment; thus, it is exploited to isolate mycobacteria (see chapter 12). The stress generated by a low pH is more severe in a nutrient-limited environment. High levels of magnesium are required for growth in mildly acidic media (Cotter 2003).

The microorganism also withstands very low temperatures. Its viability may be increasingly preserved for a long term between 2-4°C to -70°C. When ultrafrozen, the viability of the bacilli remains almost intact as well as the taxonomic, serologic, immunologic, and pathogenic properties. After thawing, they may require re-adaptation to recover full metabolic activity (Kim 1979). On the other hand, the bacilli are very sensitive to heat, sunlight and ultraviolet (UV) irradiation. In sputum or in aqueous suspension, they progressively lose viability between 30 and 37°C within one week. Exposed to direct UV irradiation, moderate loads of tubercle bacilli die in a few minutes (Huber 1970, Collins 1971).

In addition, *M. tuberculosis* tolerates low oxygen tension as demonstrated in undisturbed liquid culture media where the self-generated microaerophilic sediment contains non-dividing, yet viable, bacilli. The bacilli may survive for many years in this condition but need a minimal concentration of oxygen to induce the switch into a fermentative metabolism (Wayne 1982, Wayne 1984). Adaptation to microaerophilic conditions was further substantiated when it was found that, unlike aerobically-cultured bacilli, those persisting at low oxygen tension were susceptible to metronidazole, a drug that is known to be effective against anaerobic bacteria. Using transmission electron microscopy, Cunningham and Spreadbury demonstrated that the cell wall of the microorganism thickens notoriously in microaerobic and anaerobic cultures, which might be a strategy to endure oxygen depletion

(Cunningham 1998). Under these conditions, a highly expressed and ubiquitous 16 kilo Dalton protein was identified. This heat-shock protein might play a role in stabilizing the cell structures for long-term survival in the dormant state.

The tight structure of the cell wall of the tubercle bacillus is undoubtedly the shield that preserves the position and function of the metabolic and replicating machinery, even when inactive. At the same time, a succession of physiological mechanisms, which are still poorly understood, are ready to shift this machinery towards dormancy whenever necessary. This seems to be the main adaptive response of the bacilli to almost all sub-optimal or even harsh conditions, *in vitro*, *ex vivo*, and *in vivo* (see chapter 5).

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