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Morphological Characterization of *Mycobacterium tuberculosis*

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1. Introduction

It is more than 100 years since the first *Mycobacterium* was isolated by Hansen (1874). That was leprosy bacillus, *Mycobacterium leprae*, which even today is still resisting all attempts to cultivate it in the laboratory. The tubercle bacillus, *M. tuberculosis* was discovered eight years later by Robert Koch (1882). The Koch discovery was confirmed by more efficient staining models of Ehrlich (1887) and Ziehl- Neelsen (1883). 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 coccobacilli to long rods. 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 width. The possibility of morphological variations in tubercle bacilli was suggested by few investigators like Malassez and Vignal (1883), Nocard and Roux (1887), Metschnikoff (1888), Lubarsch(1899), Fischel(1893), and Vera and Rettger (1939). They showed under unfavorable conditions, i.e., a limited food supply, or oxygen deprivation, *Mycobacterium* assumed a swollen appearance without forming the vacuolar or globoid bodies (Vera and Rettger, 1939). These early reports were based on stained preparations and were subjected of severe criticism (Porter and Yegian, 1945). Today with advances in microscopic technique i.e., transmission electron microscope (TEM), scanning electron (SEM) and atomic force microcopy (AFM), almost all of investigators have been agreed that the Koch bacillus does not always manifest itself in the classical rod shape (figure 1). They become shorter in older cultures, filamentous within macrophages and ovoid during starvation (Young *et al.*, 2005; Farnia *et al.*, 2010; Shleeve *et al.*, 2011) and they may produce buds (Chauhan *et al.*, 2006) and branches in extensively drug resistance strains (XDR-TB) (Velayati *et al* 2010; Farnia *et al* 2010). In the following parts the underlying mechanisms that may help the bacilli to change its morphology was highlighted.

2. The role of cell wall in shape maintenance

The cell wall of mycobacterium is characterized by a unique structure which is caused by partly distinct chemical compositions in comparison with the cell wall of other bacteria

(Koike and Takeya, 1961; Imaeda and Ogura, 1963; Imaeda *et al.*, 1969). These variations are thought to be advantageous in stressful conditions of osmotic shock or desiccation as well as contributing to their considerable resistance to many drugs (Jarlier and Nikaido, 1990). The *Mycobacterial* cell wall, in principal, consists of an inner layer and an outer layer that surround the plasma membrane (Hett and Rubin, 2008). The outer compartment consists of both lipids and proteins (Draper, 1971, 1998; Draper *et al.*, 1998; Brennan and Nikaido, 1995; Brennan, 2003). The inner compartment consists of peptidoglycan (PG), arabinogalactan (AG), and mycolic acid (MA) covalently linked together to form a complex known as MA-AG-PG complex that extends from the plasma membrane outward in layers, starting with PG and ending with MAs. The Peptidoglycan, which forms the “backbone” of the cell wall skeleton, was first studied by Misaki *et al* (1966). It belongs to a family of structures possessed by almost all bacteria and blue-green algae but by no other type of living organism (Schleifer and Kandler, 1977); its presence in mycobacteria provides conclusive evidence that they are not, as was once believed, some sort of intermediate stage between bacteria and fungi. The peptidoglycon is made of peptides and glycan strands. The long glycan strand typically consists of repeating N-acetylglucosamines (NAGs) linked to N-acetylmuramic acid (NAM). These strands are cross linked by peptides bound to the lactyl group on NAMs from different glycan strands. These peptide chains normally consist of L-alanyl-D-iso-glutaminy-meso-diaminopimelic acid (DAP) from one strand linked to the terminal D-alanine residue from L-alanyl-D-iso-glutaminy-meso- DAP-D-alanine from a different strand (Kotani *et al.*, 1970; Wietzerbin *et al.*, 1974). This highly cross-linked glycan meshwork of PG that surrounds bacteria is the primary agent that maintains bacterial shape. The structure of this stratum differs slightly from that of common bacteria, as it presents some particular chemical residues and unusual high number of cross-links. Indeed, the degree of peptidoglycon cross linking in the cell wall of *M. tuberculosis* is 70-80%, whereas that in *E. coli* is 20-30%. (Matsushashi, 1994; nVollmer and Holtje, 2004). PG isolated from *E. Coli* retains its rod-like shape even in the absence of all other material (Weidel *et al.*, 1960; Weidel and Pelzer, 1964), confirming its role in shape maintenance. Also, treatment of bacteria with lysozyme which degrades PG, results in rod shaped cells becoming round spheroplasts (Lederberg, 1956). Spheroplasts, or round bacteria lacking PG, can be formed in *M. smegmatis* through degradation of PG. Upon transfer to growth media, the spherical bacteria are able to regenerate wild-type rod-shaped cells (Udou *et al.*, 1982). This occurs through elongation of bacteria that then branch, septate and fragment. These data argue that shape and size are not simply governed by existing PG, but there must be some genetic heritable determinant also.

3. Peptidoglycan synthesis

Little is known about the biosynthesis of the peptidoglycan of *M. tuberculosis*. However, it is generally assumed to be similar to that of *E. coli* (van Heijenoort, 1998). Generally, peptidoglycan synthesis occurs in four sequential steps. First, inside the cytoplasm, soluble substrates are activated and peptidoglycon units are build. Glucosamine is enzymatically converted into MurNAc and then energetically activated by a reaction with uridine triphosphate (UTP) to produce uridine diphosphate -N-acetylmuramic acid (UDP-MurNAc) (De Smet *et al.*, 1999). Second, at cytoplasmic membrane, the units UDP-MurNAc pentapeptide is attached to the bactoprenol “conveyor belt”, through a pyrophosphate link

with the release of uridine monophosphate (UMP)(Crick *et al.*, 2001; Yuan *et al.*, 2007). Third the bactoprenol molecule translocates the disaccharide pentapeptide precursor to the outside of the cell. The GlcNAc-MurNAc disaccharide is then attached to a peptidoglycan chain using pyrophosphate link between itself and the bactoprenol as energy to drive the reaction. The pyrophosphobactoprenol is converted back to a phosphobactoprenol and recycled. Fourth, outside the cell but near the membrane surface, peptide chains from adjacent glycan chains are cross-linked to each other by a peptide bond exchange (transpeptidation) between the free amine of the amino acid in the third position of the pentapeptide (e.g., lysine) or the N-terminus of the attached pentaglycine chain and the D-alanine at the fourth position of the other peptide chain, releasing the terminal D-alanine of the precursor (Wietzerbin *et al.*, 1974; Ghuysen, 1991).

4. Control of peptidoglycan synthesis

Enzymes involved in remodeling PG can be grouped as either biosynthetic or hydrolytic. Biosynthetic enzymes include transglycosylase and transpeptidase domains, often found on a single, bifunctional protein. Hydrolytic enzymes include muramidase, glucosaminidase, lytic transglycosylase, amidase, endopeptidase and carboxypeptidase (Young, 2003; Cabeen and Jacobs-Wagner, 2005). The reaction of these enzymes may be antagonistic, or they may physically interact to form complexes capable of breaking bonds to generate openings for new monomers, while also forming bonds necessary to unit PG strands. The production of these enzymes should be regulated, otherwise the bacterial cell wall would be degraded and the bacteria would be lysed. There are several ways to governate these enzymes; one method is through formation of complexes with other proteins (Hett and Rubin, 2008). These proteins could suppress the activity of the enzyme, or they could be enzymes themselves with antagonistic reactions that join rather than degrade PG. Another possibility is that the enzymes are sequestered from their substrate until they needed. A third method could be that the appropriate substrate is not made available until cleavage of it is required.

5. The role of PG in cell shape regulation

The PG -synthesizing enzyme organize into complexes that likely contributes to the resulting shape. Various models have been proposed which explain how this organization affects the bacterial shape. The two -competing sites" model (TCS) for peptidoglycan assembly advocates that, in bacterial rods, the shape depends on the activity of two biochemical reactions (sites) which occur in the terminal stages of peptidoglycan synthesis; one site is responsible for lateral wall elongation , and the other is responsible for septum formation (Lleo *et al.*, 1990; Alaedini and Day, 1999). The two sites compete with each other in such a way that the lateral wall is not extended during septum formation and *vice versa* (Lleo *et al.*, 1990; Satta *et al.* ,1994). The actual shape of the bacteria is thus determined by the balance between the two competing reactions, correct balance leading to normal rods; abnormal prevalence of the site for lateral wall elongation leads to long rods or filaments, whereas prevalence of the site for septum formation leads to formation of coccobacilli or cocci (Lleo *et al.*, 1990). The other bacteria carry only one site for peptidoglycan assembly which can form only septa and can grow only as cocci. Another model is "three -for -one " predict the insertion of PG along a track, using an existing strand of PG as a template

(Holtje, 1998). This results in doubling the length in one direction, but since following a strand, no additional length is added in the direction perpendicular to the strand. Thus width would stay constant. Another theory as to how cells maintain a constant width posits that the poles are capped with a type of PG that prevents rapid turnover or insertion of new PG (De Pedro *et al.*, 1997). Thus, the caps would restrict the width of the bacterium.

6. How the shape remains constant

Uniform cell shapes are favored by the need to segregate the chromosome and cytoplasmic material between daughter cells (Errington *et al.*, 2003). The regular shape would seem to be the best way to ensure each daughter, because a symmetrical cell can be halved accurately by mechanisms that measure length or volume (Helmstetter *et al.*, 1990; Young, 2006). In an irregular cell, misplaced septation might leave one cell with both chromosomes or with more than its fair share of other components. Therefore, once a particular shape is adapted, bacteria have a vested interest in keeping it (Stewart, 2005). The major incentive for doing so is to maintain a consistent relationship between cytoplasmic volume and surface area so that cell cycle events can be coordinated properly. This is visualized by considering the septation event that creates two daughter cells (Harry, 2001; Errington *et al.*, 2003). The septum is formed through the in-ward growth of cytoplasmic membrane and cell wall material that invaginates from opposing directions at the central plane of the cell. In such case, the concentration of essential division proteins will not change, but the surface area over which they must act will be greater in the sphere. The amounts of these proteins, if optimized for dimensions of a rod, might not be sufficient to initiate or complete normal septation and division in a coccus (Young, 2006). Thus limited concentrations of division proteins will dictate that the cell maintain a specific and constant diameter. To do this, bacteria must coordinate events spatially and temporally. Recently it was shown that the divisome will assemble at midcell, before chromosomes are partitioned. The divisome consists of a set of 10 to 15 proteins that are required to the middle of the cell and are responsible for generating the septum that divides two daughter cells (Margolin, 2006; Buddelmeijer and Beckwith, 2002). This is accomplished by synthesizing septal PG, constricting the cell wall to eventually close off the cytoplasmic compartments of each daughter cell, and finally hydrolyzing part of the PG that holds two together in order to physically separate the cells. These divisome proteins (FtsA, FtsB, FtsE, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, FtsX, FtsZ, ZipA, AmiC and EnvC) encoded in different bacterial genomes and have different functions (Di Lallo *et al.*, 2003; Karimova *et al.*, 2005; Vicente and Rico, 2006). The FtsZ is the first protein to assemble at midcell (Bi and Lutkenhaus, 1992). Its formation of a ring around the cell, just under the plasma membrane, gives the assembled divisome the name Z ring. This sub-cellular organelle, a functional analog of the contractile ring used in cytokinesis of many eukaryotic cells, is thought to form the scaffold for recruitment of the other key cell division proteins. In *E. coli*, successful cell division depends on a constant and critical concentration of FtsZ combined with proper proportions of Z-ring stabilizing and destabilizing proteins. Significantly, small changes in the concentrations of FtsZ or other essential division proteins disrupt cell growth. Thus, division is inhibited if FtsZ is under-produced, extra divisions occur if the protein is over-produced and no division occurs if FtsZ levels are adequate but FtsZ/FtsA ratio is incorrect (Errington *et al.*, 2003; Maki *et al.*, 2000; Chauhan *et al.*, 2006).

7. Shape variation

The tubercle bacillus is a 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), metabolically flexible bacterium (Edson, 1951; Ramakrishnan *et al.*, 1972; Niederweis, 2008). The success of tubercle bacilli as a pathogen can be attributed to its extraordinary capacity to adapt to environmental changes throughout the course of infection. Generally, the nutritional quality and physical conditions will determine the temporary lifestyle of bacillus. These changes include: nutrient deprivation, hypoxia, temperature, PH, salinity and various exogenous stress conditions (Vera and Rettger, 1939; Smeulders *et al.*, 1999; Honer *et al.*, 2001; Young *et al.*, 2005; Anuchin *et al.*, 2009; Velayati *et al.*, 2009; Farnia *et al.*, 2010; Singh *et al.*, 2010; Shleeve *et al.*, 2002, 2010). Unfortunately, in most of cases we do not know if shape *per se* is beneficial, because few experiments have addressed the question. 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. Recent advances in microscopy techniques have revealed adaptive changes in size and shape of bacilli under

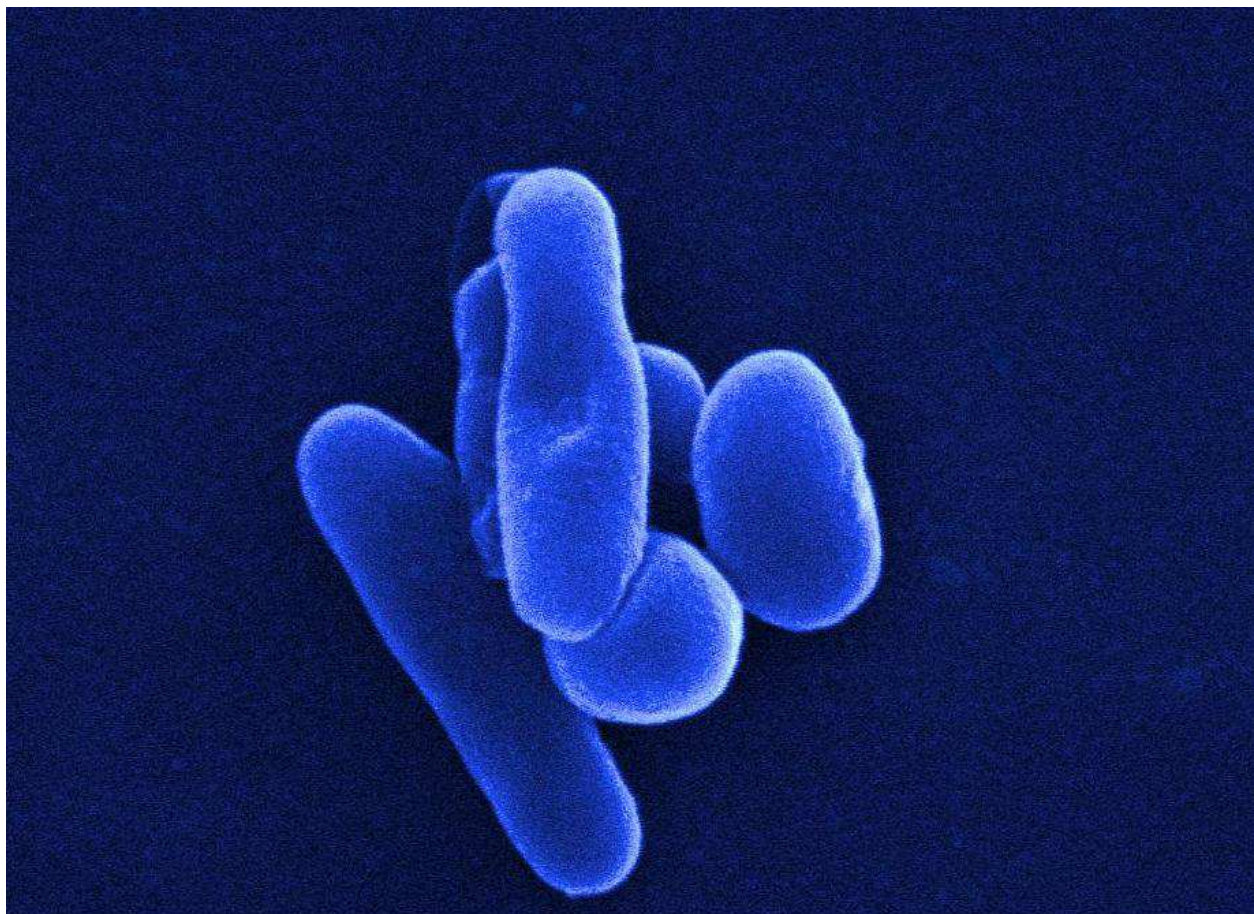


Fig. 1. Scanning electron microscope shows shape variation in *M. tuberculosis* at exponential phase of growth.

stress conditions (Velayati *et al.*, 2009,2011; Farnia *et al.*, 2010). Briefly, the reported morphological variation in *M. tuberculosis* are classified into two categories; those which frequently seen at exponential phase of growth that is rod, V, Y-shape, branched or buds, and those that are seen occasionally under stress or environmental conditions which are round, oval, ultra-virus, spore like, and cell wall defiant or L-forms.

8. Shape variations during active or exponential phase of growth

The most classical form of tubercle bacilli is a slender rod shape that seen in stained smears. They have smooth, homogenous cytoplasm with clear-cut and well-define outlines. The first electron microscope images of the tubercle bacilli were obtained in 1939 in the laboratories of the Technische Hochschule, Berlin.

von Borries and E. Ruska (1939) published electron micrographs of the avian strain of tubercle bacilli magnified 26,000 times. The cytoplasm of these bacilli contained dark bodies of different sizes. Later on, Lembke and Ruska (1940), culture the bacilli on petragrani medium and observed up to eight large bodies inside the cytoplasm of bacilli. Rosenblatt, Fullam and Gessler (1942) in their studies of tubercle bacilli in the electron microscope, confirmed many earlier observation and added some new data, particularly concerning the internal structure of bacilli. The bacilli varied in size. The size of the strain H₃₇ sub-cultured at Columbia University varied from 4.3 μ X 0.4 μ to 1.0 μ X 0.2 μ . The cell wall was always present (sometimes it was as thick as 0.03 μ) and contained granules. The internal structure showed dense nuclear masses within the granular cytoplasm. The density of the cytoplasm varied; it contained many granules and vacuoles of different sizes. Later on it became clear that the cytoplasm of young cells is dense, the basic dyes stain it deeply and uniformly, and it contains vacuoles and hyper chromic bodies. The cell protoplast was seen surrounded by a 0.023 μ thick and ductile cell wall. The cytoplasm itself was covered with a thin cytoplasmic membrane which closely adhered to the cell wall (Rosenblatt *et al.*, 1942; Knaysi *et al.*, 1950; Werner, 1951; Draper, 1982). In rod like bacilli, the process of cell division resembles that of most grams -positive bacteria (figure, 2). In the equatorial zone of the cell, on the inner side of the cell wall, a double cell plate was formed. The growth of this plate proceeded till the mother cell wall was divided into two daughter cells. The separation of newly formed cells occurred between these plates, which then covered the poles of the right and left cells. Before the cytoplasm divided, the division of cellular bodies was observed (Edwards, 1970; Nishiura *et al.*, 1970; Dhal, 2004).

The other types of cell shape (V or Y - shape bacilli) occurs in lower frequency (Dahl, 2004; Farnia *et al.* 2010). The V-shape bacilli are caused by snapping post-fission movements (Krulwich and Pate, 1971). The term “snapping division” was first described by Kurth (1898) and has been reported by many other investigators. Upon completion of cell division, one or both of the two daughter cells suddenly swing around, bringing their distal ends closer together while still remaining attached by a small region at their proximal ends. The exact mechanism responsible for snapping postfission movements is not clear. Bisset (1955) claimed that all so-called postfission movements were nothing but artifacts due to mechanical stress on the dividing cells (e.g., cells growing between solid agar and a cover slip) and would not occur if the same cells were grown in liquid cultures. Sgueros (1957)

suggested that V-forms resulted from “germ tube extrusions” from each of a pair of attached arthrospores and were not due to postfission movements. More studies have demonstrated that snapping division or V-forms could arise by any of three methods: (I) germination of adjacent coccoid elements, (ii) subpolar germination (budding) of rods, and (iii) snapping postfission movements (Starr and Khan, 1962). In mycobacterium, during septum formation the plasma membrane and inner cell wall grow inward but the outer cell wall layer remains intact. Upon completion of septum formation with a cross-wall, the inner layer may continue to grow and thus exert pressure upon the outer cell wall layer. The outer layer eventually ruptures first on one side of the cell, and the two daughter cells bend in on the side where the outer layer is still intact forming a “V-form (Dahl,2004; Farnia *et al*,2010; Malhotra *et al.*, 2010)

Mycobacterium is known to form a “Y-shaped” cells with branches more interior to the cells and of greater length figure 3. Brieger *et al* in 1954, was among the first scientist who demonstrate the branching in the reproductive cycle of *M. avium*. He showed that young culture of bacilli when first transplanted to fresh medium it consists mainly of short coccoid rods. These elongate into filaments (8-10 μ) which continue to divide and grow during a phase of filamentous proliferation. The filaments usually have two fully

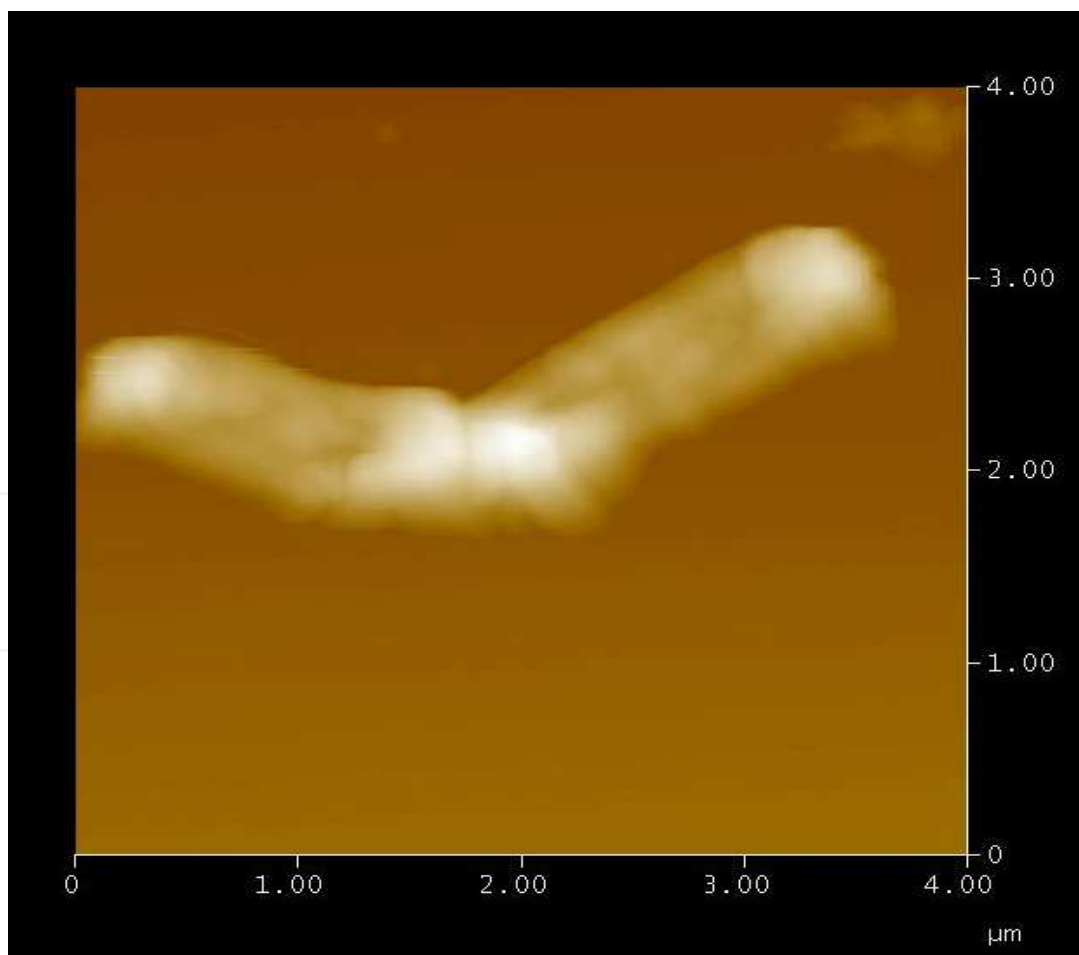


Fig. 2. Atomic force microscopy shows the V-shape *M. tuberculosis* during exponential phase of growth

developed dense bodies in polar positions and in some organisms a number of smaller are also seen scattered among the cellular units and apparently associated with them. The final stage in the reproductive cycle led to a massive production of small rods. At this phase the filaments suddenly break down into masses of short rods which elongate to form the new generation and the cycle is complete. Under electron microscope, it was seen that the filaments were quite separate, and there was no true branching and that the mycelia appearance was produced because the filaments often remained stuck together. In another study, Mizuguchi Y *et al* (1985) showed β -Lactam antibiotics at low concentration induced filamentous cells in the *M. avium-intracellular* complex. Although, the mechanisms of induction of filamentous cells appeared to be different according to the drugs used. Ampicillin induces filaments by inhibiting the septation in a manner similar to its effect on *E. coli*, whereas cephalosporin induces filaments but does not inhibit septation. In *M. tuberculosis*, branches were first seen as a small bud that does not grow to any appreciable size before breaking off as a separate cell. Few studies suggested that *M. tuberculosis* grows from the ends of bacilli and not along the length of the cylinder as seen in other well-characterized rod shape bacteria (Thanky *et al.*, 2007). This might be true for susceptible isolates, but recently Farnia *et al* (2010) showed that in highly drug resistance strains i.e., XDR-TB and Totally or Extremely drug resistant isolates (TDR or XXDR-TB), branches produce along the cylinder. In fact, about 20 -24% of cells in XDR and XXDR-TB bacilli were dividing by branching, respectively.



Fig. 3. Transmission Electron Microscopy shows Y-Shape *M. tuberculosis* at exponential phase of growth

9. Cell shapes during dormancy or under limited conditions

The morphological variations in tubercle bacilli become evident when the culture medium was poor. These changes were first reported by Koch himself. In his paper on the “discovery

of the cause of tuberculosis", he described that "under certain conditions, some bacilli contain several spores, in most cases there are two to four of them; oval in form, they are distributed, in uniform intervals, along the axis of the bacilli(1882). Following Koch discovery, Malassez and Vignal (1883) had described, the small "coccoid bodies " which cause tuberculosis infection and named them as cell wall deficient forms (CWD-forms) of tuberculosis. Later on, Spengler (1903, 1905), were among the first scientist who could demonstrated that in older cultures and frequently in sputa, apparently in response to adverse environmental conditions, the smooth cell takes on a fragmented appearance. Much (1907) was able to reproduce granules in the inside of the bacilli as well as scattered around them. These granules, according to Much, cannot be stained by the Ziehl- Neelsen technique but may generate new tubercle bacilli. Later on 1909, Fontes revealed how he had applied double staining to the bacilli, namely Ziehl- Neelson's carbol-fuchsin staining and the Gram treatment. In this way he tried to differentiate the pathogenic tubercle bacilli, containing Much granules, from the apathogenic ones without these granules. In 1910, Fontes described the multiplication through division of these granules in the inside of a cell and on its outside and applied the term "virus" to this formation. Fontes described the application to the tubercle bacillus of the well -known method of separating the virus from the substrate by filtering the material through a bacterial filter. He inoculated a guinea pig with the filtered caseous material and transplanted the organs of this animal into a fresh one. When after five months of observation the animal was killed, the autopsy revealed the infiltration of round cells, granules, and occasional acid-fast bacilli in the lymph nodes and the lungs. After years of oblivion, the early works of Fontes were rediscovered by Vandremere (1923). He repeated the Fontes filtration experiments and confirmed the development of acid -fast bacilli on media and in animals inoculated with these filtrates. Calmette (1926) advanced the theory on the role of the the tuberculosis " ultra-virus" in the development of certain forms of the diseases. However, Negre *et al* (1933) denied the existence of filterable forms of the mycobacteria. Few years later, Vera and Rettger (1939) studied four strains of *M. tuberculosis*(hominis), "Koch", 607, 75 and H₃₇ in micro-culture by Hill hanging block technique. This method was employed to permit observation of individual cells and their progeny over long periods of time using lucida drawings camera. They could demonstrate various forms which have been described in the literature at one time or another. When they cut off air supply, different variants developed very soon. The bacilli swelled slightly, the cytoplasm become less clear and smooth. The swelling commonly occurred at the ends of cells, so the clubs and dumbbell shapes were formed; cells often became spoon shaped. These swollen structures became increasingly refractive and more sharply delimited, until finally there was a definite superficial resemblance to spores. At the similar time, the ability of the tubercle bacillus to survive environmental hardship in culture was documented by Corper and Cohn in a study published in 1933. In another study, McCune and other colleagues (1965, 1966), showed the capacity of tubercle bacilli to survive in mouse tissue after sterilization. In this model, out bred mice were infected intravenously with 10⁵ colony-forming units of the H₃₇R_v strain of *M. tuberculosis*. They were immediately treated for a period of 12 weeks with the antimycobacterial drugs isonizid (INH) and pyrazinamide (PZA). For 4-6 week period after withdrawal of therapy, the mice showed no evidence of cultivable tubercle bacilli (sterile state). But, 12 weeks after INH and PZA treatment was withdrawn, one-third of the mice developed full-blown active TB, with nearly two-thirds

displaying disease after 24 weeks. Csillag (1962, 1963, and 1964) considered Mycobacteria as dimorphic organisms in the same sense as are some pathogenic fungi, for instance, *Histoplasma capsulatum*. The usual acid fast form of the mycobacteria was termed 'form 1' and the form which was not acid fast was termed 'form 2'. When form 2 grown in digest broth, form 2 strains produced cocci which continued to multiply by binary fission and bud formation (Csillag, 1964).

These forms were not produced by mycobacteria grown in rich media such as nutrient broth; Martin's digest broth, yeast extract and Lab-Lemo beef extract. One year later, Stewart-Tull (1965) isolated two forms of mycobacteria and mycococci from *M. phlei*. Nyka W in 1963, described them as "chromophobic tubercle bacilli" in the lungs of patients treated by drugs in association with surgery. This organism morphologically were similar to the acid- fast bacilli, but do not stain with either carbolfuchsin or the counter stains when applied by the classic Ziehl-Neelsen technique or with any other aniline dye. In continuation of his work, he submitted the culture of *M. tuberculosis*, *M. kansasii*, and *M. phlei* to starvation. As a result they lost first their acid fastness, but in this chromophobic state, they survived for at least 2 years, and after that time, produced cultures of acid fast bacilli when transferred onto nutrient media. Since these *in-vitro* bacilli could recover their original biological properties, it was concluded that those bacilli in the lung could also become reactivated and cause a relapse of the disease. Some scientists regard the filterable forms of mycobacteria as being analogous to the so -called L-forms of the other bacterial genera as they also pass through filters (Thacore and Willett, 1963). Some other scientists believe that development of the L-form is a mutation process, while development of the filterable forms is an adaptation of the micro-organisms to enable them to multiply in unfavorable (Imaeda, 1974; Mattman, 1970: Ratnam and Chandrasekhar, 1976). In this regards, Takahashi (1979), reported that tubercle bacilli in caseous lesions seems to be non acid fast, gram negative granules which may revert into acid fast rods, when the caseous lesion begins to liquefy and form tuberculous cavity. Similarly, khomenko and colleagues (1987) showed ultra-fine forms of *M. tuberculosis* in the walls of open cavities in the lungs of experimental animals by electron microscopy. These invisible forms of *M. tuberculosis* are able to revert to the typical bacterial forms. The initial stage of this process is accompanied by the formation of coccoid forms of mycobacteria that can be detected when material is inoculated on to semi-synthetic medium with 10% plasma and by microscopy of the sediment. Lawrence Wayne (1994) postulated that bacilli recovered from granulomatous lesions had adapted to a relatively oxygen starved environment so that they would be unable to grow in an aerated culture and therefore, may be non-cultivable by traditional culture methods (Wayne and Hayes, 1996). In the Wayne model, cultures of the bacterium are subjected to gradual self-generated oxygen depletion by incubation in sealed stirred tubes. Upon the slow shift of aerobic growing *M. tuberculosis* to anaerobic conditions, the culture is able to adapt and survive anaerobiosis by shifting down to a state of nonreplicating persistence. Wayne L showed two phase of growth in mycobacterium under limited oxygen; initially when the level of drops and the turbidity increased in culture tubes (NRP-1) and in anaerobic phase when there is no oxygen and no division (NRP-2). Wayne model was a break through in understanding what may happen to tubercule bacilli in necrotic material (Wayne and Lin, 1982). Although, Kaprelyants *et al* (1993) did not consider the bacilli

obtained by Wayne and Sramek (1994) as dormant because they maintained a high viability and developed sensitivity to metronidazole when anaerobic, thus indicating active metabolism. Therefore, from large accumulated data that found in literature, it become clear that *M. tuberculosis* can adapt rapidly to changing environment inside and outside the host (Parrish *et al.*, 1998; Cardona, 2009; Rustad *et al.*, 2009). These capacities will allow the tubercle bacilli to survive for long time in a dormant state in the lung tissue. Recently, Peyron *et al* (2008) developed an *in vitro* model of human tuberculosis granulomas. In this model granuloma-specific cell types and their modulation by tubercle bacilli were characterized. More recently, the complete morphological changes that occurs in tubercle bacilli under hypoxic conditions viewed under AFM (every 90 days for 48 months) (Velayati *et al.*, 2011). The morphological adaptation classified into two categories; First was temporary adaptation (from 1 to 18 months of latency) in which cells undergoing thickening of cell wall (20.5 ± 1.8 nm versus 15.2 ± 1.8 nm, $P < 0.05$), formation of ovoid cells by “folding phenomena” (65-70%), size reduction (0.8 ± 0.1 μ m versus 2.5 ± 0.5 μ m), and budding type of cell division (20-25%) (figure 4).

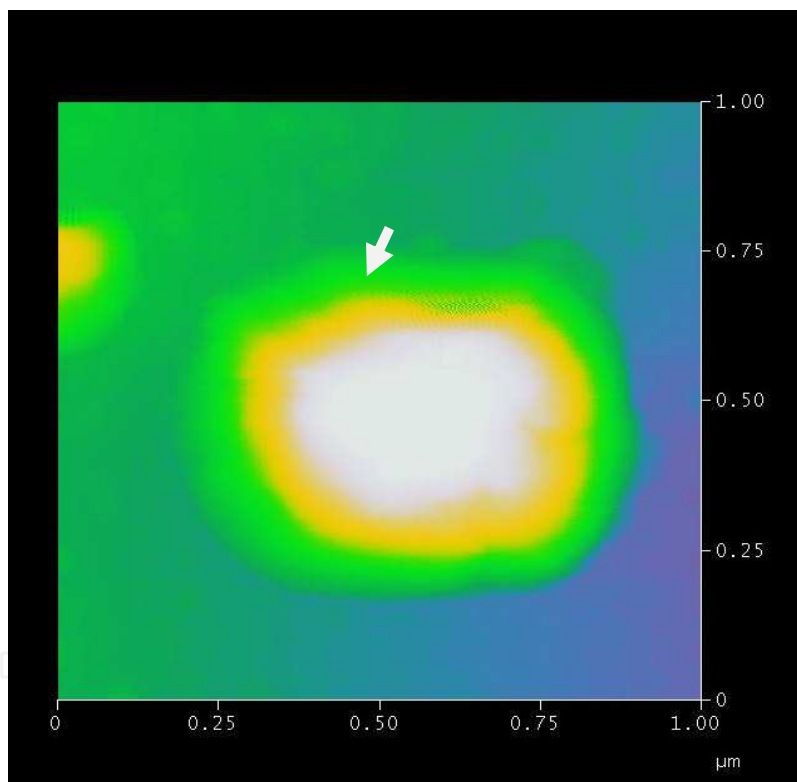


Fig. 4. Atomic force Microscopy shows *M. tuberculosis* under 8 months hypoxic condition. The bacilli becomes round and developed a thickened cell-walls (shows by arrows)

A second feature include changes that accompany development of specialized cells (from 18 to 48 months of latency) i.e., production of spore like cells (0.5 ± 0.2 μ m) and their progeny (filterable non -acid fast forms; 150 to 300 μ m in size figure 5). Using AFM, they could demonstrate that the filterable non-acid fast forms of bacilli are produced from spore -like cells. These cells were metabolically active and increased their number by symmetrical typing of division and could be stain by gram staining. Inoculation of these cells could induce active tuberculosis in mice. Although, it is important to determine how closely the

in vitro models correlate to the state of *M. tuberculosis* during latent infection. But, if these models are predictive of human disease, the information they provide in combination with advances in animal models, imaging and analysis will substantially aid in the development of drugs capable of killing tubercle bacilli in altered metabolically states, and possibly shortening the course of TB therapy.

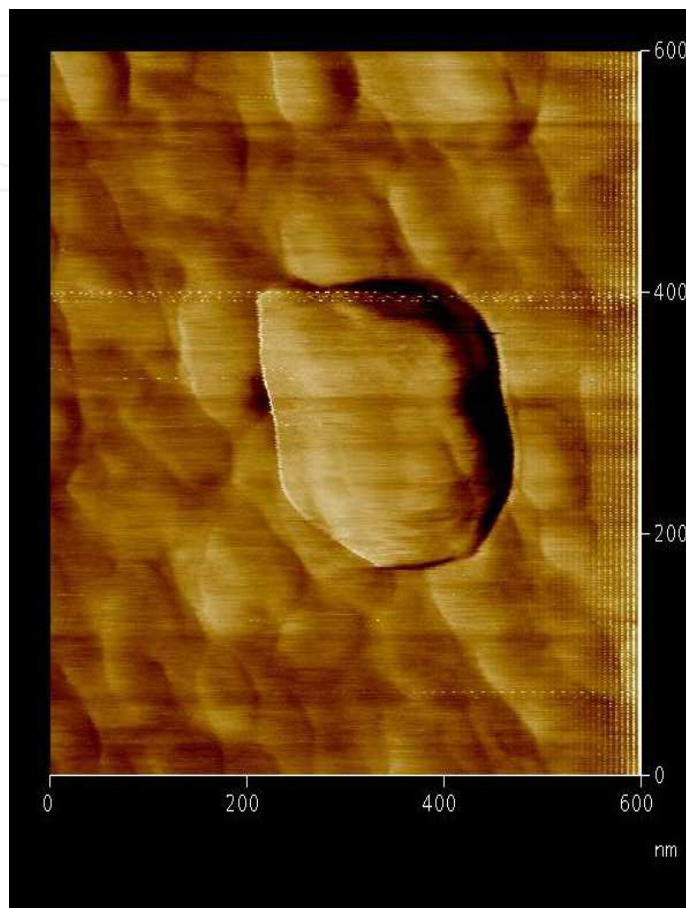


Fig. 5. Atomic force microscopy shows the Latent TB bacilli, after 48 months of latency (Velayati *et al.*, 2011).

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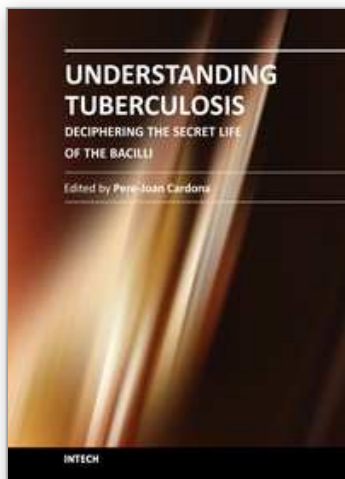
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Mycobacterium tuberculosis, as recent investigations demonstrate, has a complex signaling expression, which allows its close interaction with the environment and one of its most renowned properties: the ability to persist for long periods of time under a non-replicative status. Although this skill is well characterized in other bacteria, the intrinsically very slow growth rate of Mycobium tuberculosis, together with a very thick and complex cell wall, makes this pathogen specially adapted to the stress that could be generated by the host against them. In this book, different aspects of these properties are displayed by specialists in the field.

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