Review Article

Shape variation in Mycobacterium tuberculosis

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Summary

Over several decades, morphological variation of *Mycobacterium tuberculosis* (*M. tuberculosis*) has engaged the attention of numerous investigators. The single point on which all investigators have agreed is that tubercle bacillus does not always manifest itself in the classical rod shape. While most commonly the organism appears as a granular rod, the other forms i.e., coccid, filament and club shapes are also present. Aside from the more purely academic aspect of the subject, the possible significance of variant forms in the etiology, prognosis, and control of tuberculosis infection were objects of heated controversies, even before 1900. These differences have never been resolved, and have been ignored by most recent workers. The main questions were centered on the following points: (1) Dose the tubercle bacillus produce endospore? (2) Does it normally undergo a complicated life cycle? (3) What is the importance of the non-acid-fast forms? (4) And what happens to the bacteria during latent infection? Today, based on various *in-vitro* and *in-vivo* models, the researchers agreed to consider *M. tuberculosis* as a two-phase microorganism which can appear either in its metabolically active acid-fast or in its inactive forms. It is the purpose of this chapter to review and discuss morphological variation and its challenges in *M. tuberculosis*. Furthermore, the cell shape and cell division were illustrated using atomic force microscopy. The present information will discuss the adaptation mechanism in *M. tuberculosis* and may help scientists to identify targets for novel therapies.

Introduction

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 (1 -3). 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 (4-12). Unfortunately, in most cases, we do not know if shape *per se* is beneficial to its adaptation capability, because few experiments have addressed the question. Knowledge of the physiology of *Mycobacterium tuberculosis* (*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 stress conditions (10, 13-4). 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.

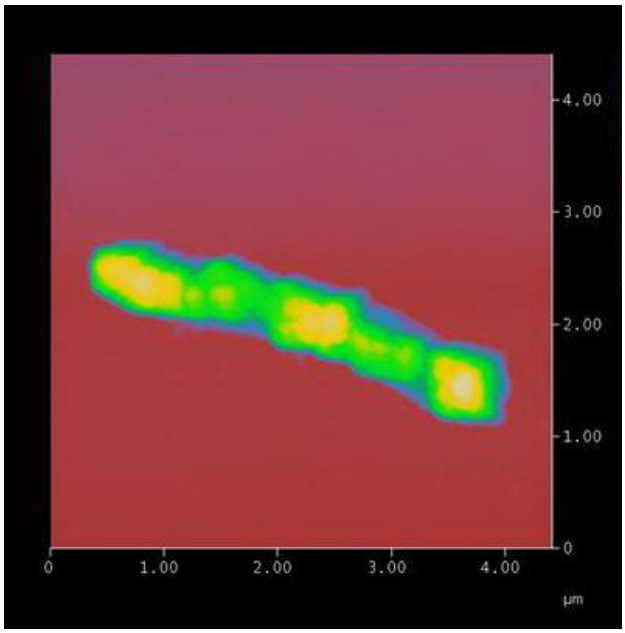

Figure 1. Atomic Force microscopy shows *M. tuberculosis* at exponential phase of growth.*(photo by Farnia P.)*

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 (figure1). The first electron microscope images of the tubercle bacilli were obtained in 1939 in the laboratories of the Technische Hochschule Berlin (15).
Von Borries and E. Ruska (15) published electron micrographs of the avian strain of tubercle bacilli

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magnified 26,000 times and the cytoplasm of these bacilli contained dark bodies of different sizes. Later on, Lembke and Ruska (16), culture the bacilli on petragnani medium and observed up to eight large bodies inside the cytoplasm of bacilli. Rosenblatt, Fullam and Gessler (17) in their studies of tubercle bacilli in the electron microscope, confirmed many earlier observations and added some new data, particularly concerning the internal structure of bacilli.

Apart from that, the bacilli varied in size. The size of the strain H_{37} sub-cultured at Columbia University varied from 4.3µ X 0.4µ to 1.0µ X 0.2µ. The cell wall was always present (sometimes it was as thick as 0.03µ) and contained granules. The internal structure showed dense nuclear masses within the granular cytoplasm and the density of the cytoplasm varied which contained many granules and vacuoles of different sizes. Subsequently, 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 0.023µ thick and ductile cell wall and the cytoplasm itself was covered with a thin cytoplasmic membrane which closely adhered to the cell wall (17-20). 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 is formed. The growth of this plate proceeds until the mother cell wall is divided into two daughter cells. The separation of newly formed cells occurs between these plates, which then covers the poles of the right and left cells. Before the cytoplasm divides, the division of cellular bodies will be observed (21-23).

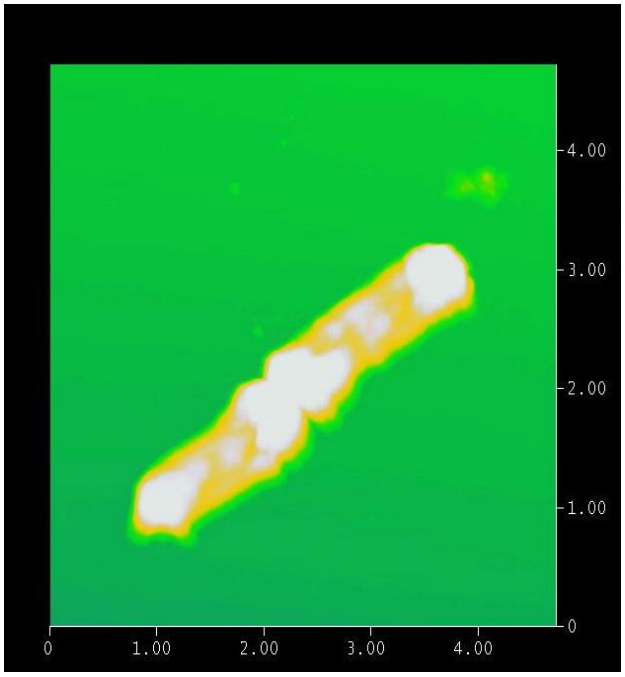

Figure.2: Atomic force microscopy shows the *M. tuberculosis* at Symmetrical type of cell division. *(photo by Farnia P.)*

The other types of cell shape (V or Y - shape bacilli) occur in lower frequency (10,23). The V-shape bacilli are caused by snapping post-fission movements (24). The term "snapping division" was first described by Kurth and has been reported by many other investigators (25).. Upon completion of cell division, one or both two daughter cells suddenly swing around, bring 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 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 (26).. Sguros suggested that V-forms resulted from "germ tube extrusions" from each of a pair of attached arthrospores and were not due to postfission movements (27).. 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 (28). In mycobacterium, during septum formation, the plasma membrane and inner cell wall grow inward but the outer cell wall layer remains intact (figure 3). 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 (10,23,29).

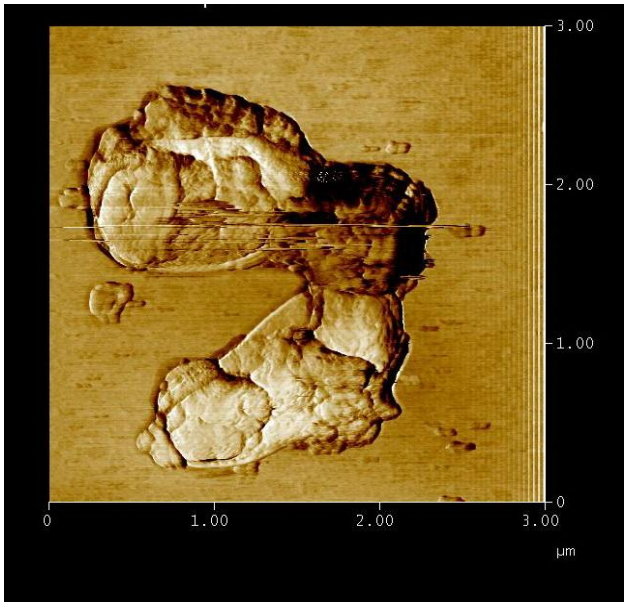

Figure 3: Atomic force microscopy shows the V-shape *M. tuberculosis* during exponential phase of growth (*photo by Farnia P.)*

Mycobacterium is known to form Y-shaped "cells with branches more interior to the cells and of greater length. Brieger *et al* in 1954, was among the first scientists who demonstrated the branching in the reproductive cycle of *M. avium* (30). He showed that young culture of bacilli, when first transplanted to fresh medium, consists mainly of short coccoid rods. These elongate into filaments (8-10µ) which continue to divide and grow during a phase of filamentous proliferation. The filaments usually have two

fully developed dense bodies in polar positions and in some organisms, a number of smaller ones 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 the mycelia appearance was produced because the filaments often remained stuck together. In another study, Mizuguchi Y *et al* showed β-Lactam antibiotics at low concentration induced filamentous cells in the *M. avium-intracellular* complex (31). However, 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 cefazolin induces filaments without inhibiting the 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 (32). This might be true for susceptible isolates, but recently Farnia *et al* (10) showed that in highly drug-resistant strains, i.e., XDR-TB and Totally or extremely drug resistant isolates (TDR or XXDR-TB), branches were produced along the cylinder. In fact, about 20 -24% of cells in XDR and XXDR-TB bacilli were dividing by branching, respectively. The Y-shape or budding cells are induced at lower rate in susceptible TB bacilli (figures 4 and 5).

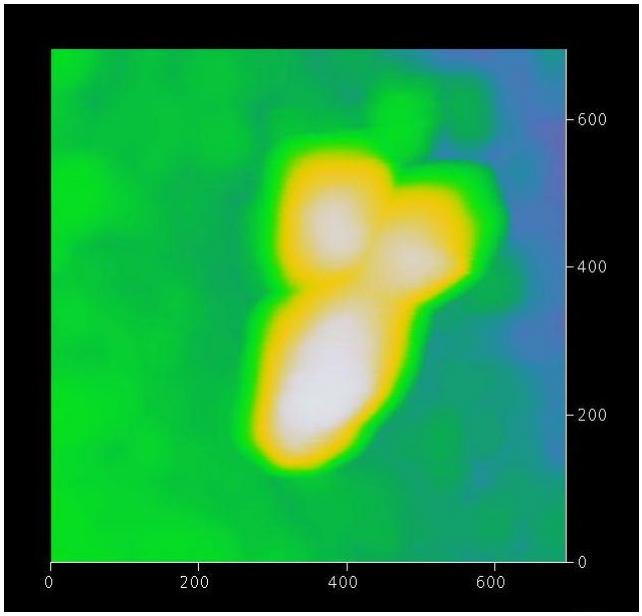

Figure 4: Atomic force Microscopy shows Y-Shape *M. tuberculosis* at exponential phase of growth (*photo by Farnia P.)*

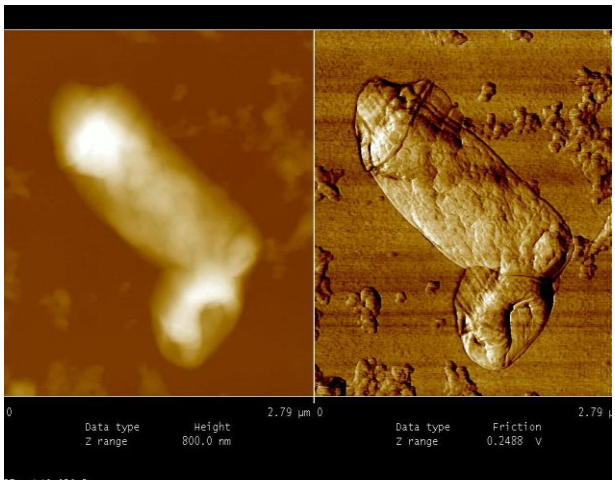

Figure 5: Atomic Force Microscopy shows buding in *M. tuberculosis* at exponential phase of growth *photo by Farnia P.)*

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 (33). Following Koch discovery, Malassez and Vignal had described, the small "coccoid bodies "which cause tuberculosis infection and named them cell wall deficient forms (CWD-forms) of tuberculosis (34). Furthermore, Spengler were among the first scientists who demonstrated that in older cultures and frequently in sputa, apparently in response to adverse environmental conditions, the smooth cell takes on a fragmented appearance (35,36).. Much was able to reproduce granules in the inside of the bacilli as well as scattered around them (37). These granules, according to his study, cannot be stained by the Ziehl- Neelsen technique but may generate new tubercle bacilli.

Later on, Fontes revealed how he had applied double staining to the bacilli, namely Ziehl-Neelson's carbol-fuchsin staining and the Gram treatment (38).. In this way, he tried to differentiate the pathogenic tubercle bacilli, containing many granules, from the apathogenic ones without these granules. Afterwards, in 1910, Fontes described the multiplication through division of these granules inside and outside of a cell and applied the term "virus" to this formation (39). . He 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. Additionally and after years of oblivion, the early works of Fontes were rediscovered by Vandremer (40). He repeated the Fontes filtration experiments and confirmed the development of acid-fast

bacilli on media and in animals inoculated with these filtrates. Calmette advanced the theory on the role of the tuberculosis "ultra-virus" in development of certain forms of the diseases (41) . However, Negre *et al* denied the existence of filterable forms of the mycobacteria (42).

Few years later, Vera and Rettger studied four strains of *M. tuberculosis* (hominis), "Koch", 607, 75 and H37 in micro-culture by Hill's hanging block technique (4). 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 soon after. The bacilli swelled slightly, the cytoplasm became less clear and smooth. The swelling commonly occurred at the ends of cells, so the clubs and dumbbell shapes were formed and 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 (44). In another study, McCune and other colleagues (45), showed the capacity of tubercle bacilli to survive in mouse tissue after sterilization. In this model, our bred mice were infected intravenously with 105 colony-forming units of the $H_{37}R_v$ strain of *M. tuberculosis* and immediately treated for a period of 12 weeks with the antimycobacterial drugs isoniazid (INH) and pyrazinamide (PZA). For 4-6 week period after withdrawal of therapy, the mice showed no evidence of cultivable tubercle bacilli (sterile state), however, 12 weeks after INH and PZA was discontinued, one-third of the mice developed full-blown active TB, with nearly two-thirds displaying disease after 24 weeks. Csillag (46-48) 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 is grown in digest broth, form 2 strains produced cocci which continued to multiply by binary fission and bud formation (48).

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 (49) 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 (50).. This organism morphologically was similar to the acid-fast bacilli but does not stain with either carbol-fuchsin 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 thereafter produced cultures of acid-fast bacilli when transferred onto nutrient media (51).

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 regarded 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 (52). Some others, however, 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 under unfavorable (53-54). In this regards, Takahashi, reported that tubercle bacilli in caseous lesions seem 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 (56).. Similarly, khomenko and colleagues (57) 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 the material is inoculated on to semi-synthetic medium with 10% plasma and by microscopy of the sediment. Lawrence Wayne (58) 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 (59). 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 non-replicating persistence. He showed two phases 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's model was a breakthrough in understanding what may happen to tubercle bacilli in necrotic material, Kaprelyants *et al* (60) did not consider the bacilli obtained by Wayne and Sramek (61) as dormant because they maintained a high viability and developed sensitivity to metronidazole when anaerobic, thus indicating active metabolism.

Therefore, from large accumulated data found in literature, it becomes clear that *M. tuberculosis* can adapt rapidly to changing environment inside and outside the host (62-64). These capacities will allow the tubercle bacilli to survive for long time in a dormant state in the lung tissue. Recently, Peyron et *al* (65) 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 occur in tubercle bacilli under hypoxic conditions viewed under AFM (every 90 days for 48 months) (66). The morphological adaptation classified into two categories; First was temporary adaptation (from 1 to 18 months of latency) when thickening of cell wall (20.5±1.8 nm versus 15.2±1.8 nm, Figure 6),.), formation of ovoid cells by "folding phenomena"(65-70%), size reduction (0.8± 0.1 µm versus 2.5±0.5 µm), and budding type of cell division (20-25%) are observed.

A second feature includes 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 µm) and their progeny (filterable non -acid-fast forms; 150 to 300 µm in size) (figures 7 and 8). Using AFM, they demonstrated that the filterable non-acid-fast forms of bacilli are produced from spore-like cells (66).. These cells were metabolically active and increased their number by symmetrical typing of division and could be stained by gram staining. Inoculation of these cells could induce active tuberculosis in mice.

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

Acknowledgments

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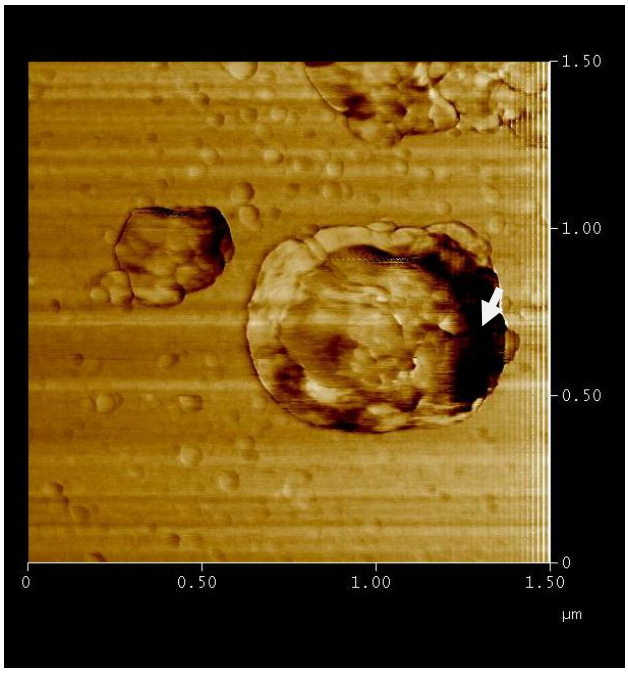

Figure 6: Atomic force Microscopy shows *M. tuberculosis* under 8 months hypoxic condition. The bacilli becomes folded and developed a thickened cell-walls (shows by arrows)

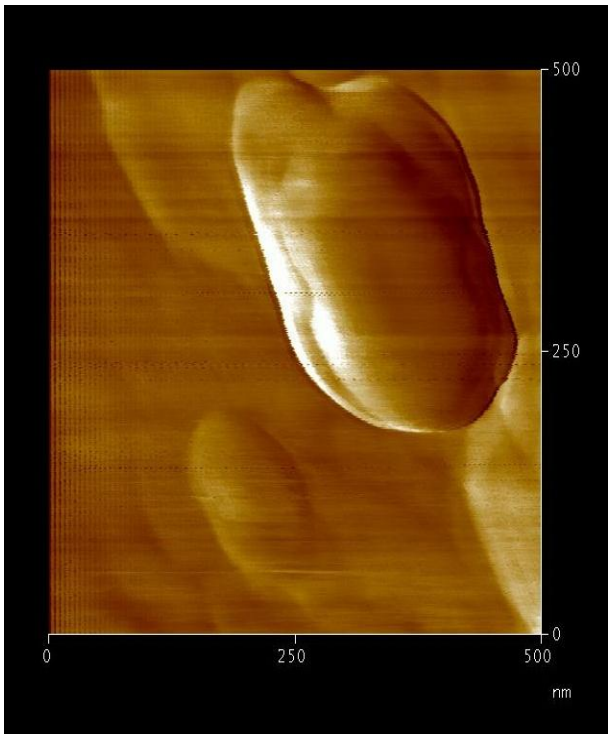

Figure 7: Atomic force microscopy shows the Spore like TB bacilli, after 24 months of latency (*photo by Farnia P.)*

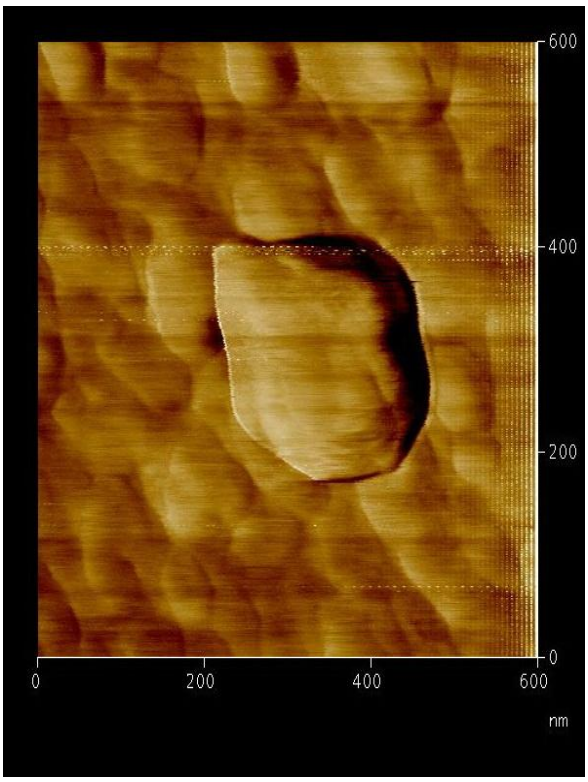

Figure 8: Atomic force microscopy shows non acid-fast TB bacilli, after 48 months of latency (*photo by Farnia P.)*

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References

1. Edson NL. The intermediary metabolism of the mycobacteria. Bacteriol Rev.1951;15:147-182.
2. Ramakrishnan T, Murthy PS, Gopinathan KP. Intermediary metabolism of mycobacteria. Bacteriol Rev.1972;36:65-108.
3. Niederweis M. Nutrient acquisition by mycobacteria.Microbiol . 2008;154:679-692.
4. Vera HD, Rettger LF. Morphological variations of the tubercle bacillus and certain recently isolated soil acid fasts with emphasis on filterability. J Bacteriol. 1940.39:659-687.
5. Smeulders MJ, Keer J, Speight RA, Williams HD. Adaptation of *Mycobacterium smegmatis* to stationary phase. J Bacteriol. 1999;181:270-283
6. Honer ZU, Bentrup K, Russell DG. Mycobacterial persistence: adaptation to a changing environment. Trends Microbiol. 2001; 9:597-605.
7. Young KD. Bacterial shape. Mol Microbiol. 2003; 49:571-580
8. Anuchin AM, Mulyukin AL, Suzina NE, Duda VI, El-Registan GI, Kaprelyants AS. Dormant forms of *Mycobacterium smegmatis* with distinct morphology. Microbiol. 2009; 155:1071-1079
9. Velayati AA, FarniaP, Ibrahim TA, Haroun RZ, Kuan HO, Ghanavi J, *et al*. Differences in cell wall thickness between resistant and nonresistant strains of *Mycobacterium tuberculosis*: using transmission electron microscopy. Chemotherapy. 2009 ;55:303-7
10. Farnia P, Masjedi MR, Farnia P, Merza MA, Tabarsi P, Zhavnerko GK, *et al*. Growth and cell –division in extensive (XDR) and extremely drug resistant (XXDR) tuberculosis strains: transmission and atomic force observation. Int J ClinExpMed . 2010; 3:320-326
11. Singh B, Ghosh J, Islam N M, Dasgupta S, Kirsebom L. Growth, cell division and sporulation in mycobacteria. Antonie van Leeuwenhoek. 2010.98:165-177.
12. Shleeva MO, Bagramyan K, Telkov MV, Mukamolova GV, Young M, Kell DB, Karprelyants AS. Formation and resuscitation of 'non-culturable' cells of Rhodococcus rhodochrous and *Mycobacterium tuberculosis* in prolonged stationary phase. Microbiol. 2002; 148:1581-1591.
13. Velayati AA, Farnia P, Masjedi MR, Ibrahim TA, Tabarsi P, Haroun RZ, *et al*. Totally drug-resistant tuberculosis strains: evidence of adaptation at the cellular level. Eur Respir J. 2009; 34:1202-1203.
14. Velayati AA, Farnia P, Merza MA, Zhavnerko GK, Tabarsi P, Titov LP,*et al.* New insight into extremely drug-resistant tuberculosis: using atomic force microscopy. Eur Respir J. 2010; 36: 1490-3.
15. von Borries B, Ruska E. VolÖufige Mitteilung uber Fortchritte im Bau und in der Leistung des Ubermikroskopes. 1938;17:99-128
16. Lembke A, Ruska H. Vergleichende mikroskopische und übermikroskopische Beobachtungen an den Erregern der Tuberkulose. 1940; 19:217-220.
17. Rosenblatt MB, Fullam EF, Gessler AE. Studies of Mycobacteria with electron microscope. Am Rev Tuberc. 1942;46:587.
18. Knaysi G, Hillier J, Fabricant C. The cytology of an avian strain of *Mycobacterium tuberculosis* studied with the electron and light microscope. 1950;60:423.
19. Werner GH. La cytologie des bacilles tuberculex etudiè en relation avec leurs caractèe de virulence. 1951; 59:1043
20. Draper P, Kandler O, Darbre A. Peptidoglycan and arabinogalactan of *Mycobacterium leprae*. J Gen Microbiol. 1987;133:1187-1194.
21. Edwards R P. Electron –microscope illustrations of division in *Mycobacterium leprae*. J Med Microbiol. 1970;3:493-499.
22. Nishiura M, Izuumi S, Mori T, Takeo K, Nonaka T. Freeze-etching study of human and murine leprosy bacilli. Inter J Lepro. 1977;45:248-254.
23. Dahl JL. Electron microscopy analysis of *Mycobacterium tuberculosis* cell division. FEMS Microbiol lett. 2004;240:15-20.
24. Krulwich TA, Pate J L. Ultra-structural explanation for snapping post-fission movements in *Arthrobacter* crystallopoietes. J Bacteriol. 1971;105:408-412.
25. Kurth H. über die Diagnose des Diphtheriebacillus unter Berücksichtigung abweichender Culturformen desselben. Z.Hyg. Infektionskr. Med.Mikrobiol. 1898.28:409-439
26. Bisset KA. The cytology and life history of bacteria, 2nd ed. The Williams &Wikins Co.,Baltimore. 1955.
27. Sguros PL. New approach to the mode of formation of classical morphological configurations by certain Coryneform bacteria. J Bacteriol. 1957;74:707-709.
28. Starr MP, Khan DA. On the origin of V-forms in *Arthrobacter atrocyaneus.* Arch Mikrobiol. 1962;42:289-298.
29. MalhotraS, Bhatia NK, KaushalM, Kaur N, Chauhan A. Pleomorphic appearance in *Mycobacterium tuberculosis*. J pub Health Epid. 2010;2:11-12
30. Brieger EM, CosslettVE, Glauert AM. Reproductive changes in Avian tubercle bacilli studied with electron microscope. J Gen Microbiol. 1954;10:294-303.
31. Mizughchi Y, OgawaM, Udou T. Morohological changes induced by B-Lactam antibiotics in Mycobacterium avium –intracellular complex. Antimicro. Agents . Chem. 1985;27:541-547.
32. Thanky NR, Young DB. Robertson B.D. Unusual features of the cell cycle in Mycobacteria: Polar –restricted growth and the snapping –model of cell division. Tubercle. 2007;87:231-236.
33. Koch R. Die Aetiologie der Tuberculose. Berlin Klinische Wochenzeitscrift 1882;19:221-226.
34. Malassez L, Vignal W. Sur le micro-organism de la tuberculose zooleique. Arch Physiol norm path III. 1883;4:81-105.
35. Spengler C. Tuberkelbacillenzüchtung aus Bakteriengemischen und Formaldehydde sinfection. 1903; 42:93.
36. Spengler C. Zur Formaldehyd-AbtÖtung und Züchtung der Tuberkel-und anderer säure-festen Bacillen. 1905; 51:335.
37. MuchH. über die granuläre,nach Ziehl nicht färbbare Forms des Tuberkulosevirus. 1907;8:85.
38. Fontes A. Estudos sobre a tuberculoze. 1909;1:51.
39. Fontes A. Bemerkungen über die Tuberkulose infktion und ihr virus.Portuguese-German text.Memorias Do Institute Oswal-do Cruz,Rio de Janiero. 1910;2:141-150. 882;19:221-226
40. Vandremer A. Formers filtrants des bacilles tuberculeux. 1923;89:80.
41. Calmette A. Existe-t-il dans la nature ou peut-on créer artificiellement des forms saprophytiques du bacilli de Koch qui soient capable de se transformer en bacilles tuberculeux virulent. 1924; 5:716-720.
42. Negre L, Valtis J, van Deinse F. Sur les caracteres biologiques des bacilles acido-resistant issus des elements filtrables du virus tuberuleux.Comptes Rendus de la Societe de la Biologie. 1933; 112:122-124.
43. Corper HJ, Cohn ML. The viability and virulence of old cultures of tubercle bacilli: studies on twelve-year broth cultures maintained at incubator temperature. Ann Rev Tuberc. 1933;28:856-874.
44. McCune RM, Feldmann FM, Lambert HP, McDermott W. Microbial persistence(I) The capacity of tubercle bacilli to survive sterilization in mouse tissues. J Exp Med. 1966; 123:445-468.

45. McCune R, Tompsett R. Fate of *Mycobacterium tuberculosis* in mouse tissue as determined by the microbial enumeration technique. J Exp med. 1956;104:737-762.
46. Csillag A. Development of form 2 mycobacterium on autoclaved Lowenstein-Jensen medium. Tubercle. 1962;43:439-433.
47. Csillag A. Cellular morphology of form 2 mycobacteria on slide culture. J Gen Microbiol. 1963;30:21-27.
48. Csillag A. The mycococcus form of mycobacteria. J GenMicrobiol. 1964;34:341-352.
49. Stewart-Tull DES. Occurrence of dimorphic forms of *Mycobacterium phlei*. Nature, London 1965;208:603-605.
50. Nyka W. Studies on *Mycobacterium tuberculosis* in lesions of the human lung. A new method ofstaining tubercle bacilli in tissue sections. Amer Rev Resp Dis. 1963;88:670-679.
51. NykaW. Studies on the effect of starvation on *Mycobacteria*. Infect Immun. 1974;9:843-850.
52. Thacore H, Willett HP. Formation of spheroplasts of *M. tuberculosis* by lysozyme treatment .Proceedings of the Society for Experiment Biology and Medicine. 1963;114:43-47.
53. ImaedaT. Ultra structure of L-phase variants isolated from a culture of *Mycobacterium phlei*. J Med Microbiol. 1974;8:389-405.
54. Mattman LH. Cell wall deficient forms of *Mycobacteria*. Ann NY Acad Sc. 1970;174:852.
55. Ratnan S, Chandrasekhar S. The pathogenicity of spheroplasts of *Mycobacterium tuberculosis*. Am Rev Respire Dis. 1976; 114: 549-54
56. Takahashi SL. Phase growth of mycobaclena.II.Consideration on the survival of tubercle bacillus in the caseous lesion. Kekkaku. 1979; 54:231-236.
57. Khomenko AG, Fadeeva NI, Golyshevskaya V I. Morphological and biochemical changes in *M.tuberculosis* during chemotherapy. Problems of Tuberculosis. 1983; 7: 48-53.
58. Wayne LG. Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur J Clin Microbiol Infect Dis. 1994; 13:908-914.
59. Wayne LG, Hayes LG. An *in vitro* model for sequential study of shift down of Mycobacterium tuberculosis through two stages of non-replicating persistence . Infect. Immun. 1996;64:2062-2069.
60. Kaprelyants AS, Gottschal JC, Kell DB. Dormancy in non-sporulation bacteria. FEMS Microbio Rev. 1993;10:271-285.
61. Wayne LG, Sramek HA. Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1994;38:2054-2058.
62. ParrishNM, Dick JD, Bishai WR. Mechanisms of latency in *Mycobacterium tuberculosis*. Trends Microbiol. 1998; 6:107-112.
63. Cardona PJ. A dynamic re-infection hypothesis of latent tuberculosis infection. Infection. 2009;37:80-86.
64. Rustad TR, Sherrid AM, Minch K J, Sherman D R. Hypoxia: a window into *Mycobacterium tuberculosis* latency. Cell Microbiol. 2009;11:1151-1159.
65. Peyron M, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, *et al.* Foamy Macrophages from tuberculosis patient's granulomas constitute a nutrient-rich reservoir for *M.tuberculosis* persistence. PLOS Pathogens.2008; 4:1-14
66. Velayati AA, Farnia P, Masjedi MR, Zhavnerko GK, Merza MA, Ghanavei J, *et al.* Sequential adaptation in latent tuberculosis bacilli: observation by atomic force microscopy (AFM). Int J Clin Exp Med 2011; 4:193-9.