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## Biofilms of *Mycobacterium tuberculosis*: New Perspectives of an Old Pathogen

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### 1. Introduction

#### 1.1 Persistence of the pathogen is the hallmark of TB pathogenesis

Based on a randomized clinical trial conducted by British Medical Council between 1972 and 1974, the World Health Organization (WHO) and other government agencies implemented a short-course multi-drug regimen for tuberculosis – a disease caused by the infection of *Mycobacterium tuberculosis* (BMC, 1972, Fox *et al.*, 1999). The regimen is made of three antibiotics, isoniazid, rifampicin and pyrazinamide administered over a period of six months. The extended therapy is essential for sterilizing a small subpopulation of bacilli that presumably acquire phenotypic tolerance to antibiotics (Saltini, 2006, Jindani *et al.*, 2003).

Four decades later, WHO estimates that about 2 billion people in the world still remain asymptotically infected with *M. tuberculosis*, approximately 5-10% of these visit clinics with symptoms of active tuberculosis, and 1.7 million die of the infection every year (Dye *et al.*, 2009). Moreover, one third of the mortality in HIV-infected patient occurs due to co-infection of *M. tuberculosis*, often with a very high frequency of multi-drug resistant strains (Harrington, 2010, Aaron *et al.*, 2004). It is thus clear that while the existing anti-TB drug regimen has been able to reduce the mortality rate, it has been inadequate in reducing the global burden of the disease. A forward approach towards TB-control must include two critical capabilities: a) to predict and prevent the conversion of asymptomatic infection to active TB, and b) to develop a shorter and more effective therapeutic regimen for active disease. Accomplishing these goals have been difficult because of our limited understanding of the mechanisms employed by *M. tuberculosis* to persist against the challenges of competent host immune system and antibiotics.

Although persistence mechanisms of *M. tuberculosis* in the host remain largely unclear, persistence of most, if not all, microbial species is facilitated by growth and existence in surface-associated and organized communities – called biofilms (Costerton *et al.*, 1999, Fux *et al.*, 2005, Hall-Stoodley *et al.*, 2004, Blankenship & Mitchell, 2006, Branda *et al.*, 2005). Several mycobacterial species including *M. tuberculosis* are now known to spontaneously grow *in vitro* as biofilms that harbor drug tolerant bacilli. This raises questions as to whether biofilms

could also be an *in vivo* persistence mechanism of *M. tuberculosis*. In this chapter we will discuss why it is reasonable to pay serious heed to this question, and what approaches can be used to test this hypothesis.

## 2. A historical perspective of studies on *M. tuberculosis* persistence

Early glimpses of the unique adaptability of *M. tuberculosis* appeared in two landmark studies conducted in the early 20<sup>th</sup> Century. First, Corper and Cohn observed that 24 out of 56 *in vitro* cultures of human and bovine isolates contained culturable tubercle bacilli even after 12 years of incubation in sealed containers (Corper & Cohn, 1933). This *in vitro* study revealed the characteristic persistence of *M. tuberculosis* in bacteriostatic condition. Concurrent with this *in vitro* study, Opie and Aronson reported the presence of virulent *M. tuberculosis* bacilli in about 26% of lesions resected from individuals dying of causes unrelated to TB (Opie & Aronson, 1927). While this study demonstrated asymptomatic infection of *M. tuberculosis*, it also opened up questions as to how bacilli are able to evade the immune system and suppress inflammation. In subsequent follow-up studies it appeared that the bacilli were unexpectedly present in uninvolved tissues instead of the presumed primary lesions (Feldman & Baggenstoss, 1939). These *in vivo* studies thus raised speculations that a competent immune system is capable of clearing the bacilli at the primary lesions, but the bacilli could have used escape mechanism to survive at secondary sites in presumably a non-replicating state.

The persistent nature of *M. tuberculosis* re-occupied the spotlight of tuberculosis research during the early phase of antibiotic-era around mid-20<sup>th</sup> Century. In several bacteriological studies on resected lesions from antibiotic treated individuals the bacilli could be microscopically observed even though individuals had converted to sputum negative (Loring & Vandiviere, 1956, Loring *et al.*, 1955, Vandiviere *et al.*, 1956, 1953). Interestingly, these bacilli in many instances were non-culturable and often associated with resolved lesions, thus raising a debate whether these were dead, or viable but non-culturable bacilli. The idea of viable but non-culturable bacilli seemed more convincing after McDermott and colleagues demonstrated reactivation of TB in mice upon termination of chemotherapy that was sufficient to reduce viability to undetectable levels (McCune *et al.*, 1966). It was, however, not clear in this study as to how and where the viable bacilli persisted, but the correlation between non-culturable bacilli and closed hypoxic lesions (Vandiviere *et al.*, 1956, Haapanen *et al.*, 1959) support the idea that closed lesions could possibly be the primary site of non-replicating persisters which developed in the bacteriostatic environment of the lesions.

Several attempts have been made to investigate the physiology of non-replicating persisters using *in vitro* models such as hypoxic and nutritionally starved cultures. These studies subsequently led to identification of genetic components responsive to these conditions—most notably *isocitrate lyase (icl)* of the glyoxalate shunt pathway and the two-component regulatory system *dosR-dosS* (Park *et al.*, 2003, Hobby & Lenert, 1957, Wayne & Hayes, 1996, Wayne & Lin, 1982, Saini *et al.*, 2004). While the mutation in *icl* impairs the persistence of bacilli in a mice model (McKinney *et al.*, 2000), the phenotype of *dosR-dosS* mutants in animal models have yielded conflicting results (Rustad *et al.*, 2008, Parish *et al.*, 2003, Malhotra *et al.*, 2004).

### 3. Changing paradigms of *M. tuberculosis* persistence

Despite the demonstration of a non-replicative and physiologically tolerant state of *M. tuberculosis in vitro* as well as the presence of hypoxic environment in granulomas (Via *et al.*, 2008), the hypothesis that the persisters in latent infection and chemotherapy are exclusively the non-replicating subpopulation residing in the bacteriostatic condition of closed lesions remains untested (Gomez & McKinney, 2004, Parrish *et al.*, 1998). In contrast, the notion of a non-replicative state of persisters during latency is strongly challenged by two interesting studies published recently. Using an unstable plasmid as a reporter, Sherman and colleagues found that *M. tuberculosis* bacilli actively replicate during the chronic phase of infection in a mouse model – a phase when neither the host develops any symptoms of disease nor the number of live bacteria changes (Gill *et al.*, 2009). Recently, Fortune and colleagues determined that mutations in *M. tuberculosis* populations accumulate at the same rate in latent and active infections of non-human primates, and both were similar to a logarithmically growing *in vitro* culture, implying active DNA replication and thus cell division of the pathogen in latent infection (Ford *et al.*).

The replicative state of the bacilli in asymptomatic infection of animal models reflects a dynamic host-pathogen interface. This interestingly is fully consistent with an emerging picture of a spectrum of disease status- in terms of bacterial load, inflammation and lesion morphologies – as against the dogmatic view of a bimodal existence of infection in either latent or active form (DB *et al.*, 2009, Rhoades *et al.*, 2005, Barry *et al.*, 2009). Interestingly, comparative studies of latent and active TB not only fail to establish a clear immunological distinction but also reveal highly heterogeneous lesion morphologies reflecting localized and highly diverse host pathogen interactions within an infected organ irrespective of the clinical symptoms (Barry *et al.*, 2009). It is thus reasonably evident that in an asymptomatic infection *M. tuberculosis* could persist in diverse physiological states – from non-replicative to fully replicative states – each with distinct host-pathogen interactions. Furthermore, persistence of actively growing bacilli in asymptomatic infection could conceivably occur through delicately balanced host-pathogen interaction, which keeps the inflammation below the symptomatic threshold, but has the greatest chance of tipping the balance to cause the active disease.

Mechanisms of persistence of *M. tuberculosis* during chemotherapy, like latency, also remains unclear, but data from clinical trials indicate a strong positive correlation between bacterial burden and duration of chemotherapy [reviewed in (Connolly *et al.*, 2007)]. Consistent with these data, the Center for Disease Control of the United States recommends an extension of chemotherapy from six to nine months in case of patients with cavitary TB (CDC, 2003). Besides the total burden, the most intriguing aspect of long-term chemotherapy in TB is that the clearance of the pathogen follows a biphasic pattern as clearly demonstrated by Mitchison and colleagues (Jindani *et al.*, 2003) (Fig. 1). While > 95% of the population could be cleared in the first few days of the beginning of treatment, the remaining fraction required a prolonged exposure (Jindani *et al.*, 2003).

In summary, the persistence of *M. tuberculosis* in a chronic infection and chemotherapy are likely to be facilitated by multiple mechanisms including the adaptive changes in the bacilli in response to dynamic microenvironments during colonization and active growth. These

changes could either be in surface structure or physiology that lead to decreased antibiotic permeability, as well as controlled host-pathogen interaction and inflammation. Therefore, addressing questions such as where and how *M. tuberculosis* colonizes during chronic infection and gaining insight into the growth phase-dependent adaptive changes are critical for a comprehensive understanding of its persistence.

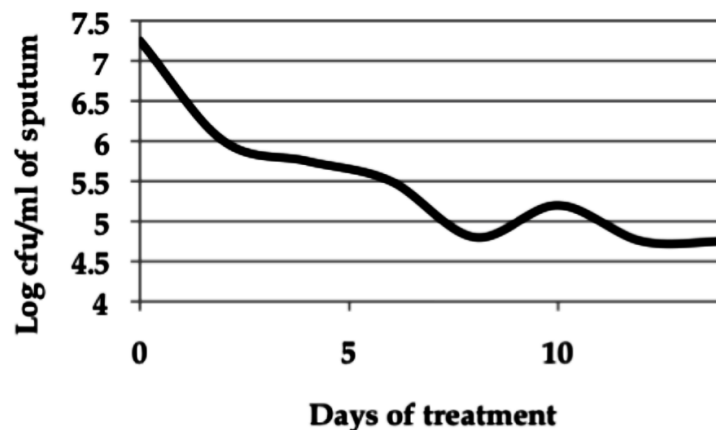


Fig. 1. Representation of the data published by Jindani et al. (4), showing the pattern of *M. tuberculosis* clearance in patients treated with isoniazid and rifampicin.

#### 4. Chronic infections, bacterial persistence and biofilms

The hallmark of a successful pathogen is to colonize the host for a long period of time against the challenges of host immune system, and persist against the antibiotics pressure. Although numerous bacterial and fungal pathogens including *M. tuberculosis* easily qualify for this category, the question as to how they establish such infections remains unanswered for most species.

However, a large and ever-growing body of evidence provide a compelling argument that the persistence of most, if not all, microbial species in general is achieved through their ability to grow in self-organized, surface associated, sessile communities called biofilms (Costerton et al., 1999, Fux et al., 2005, Hall-Stoodley et al., 2004, Kolter & Greenberg, 2006, Marrie et al., 1982, McNeill & Hamilton, 2003, Donlan & Costerton, 2002). Moreover, several long-term colonizers in humans like *P. aeruginosa*, *S. aureus*, *S. epidermis*, *C. albicans*, *H. influenzae* and *E. coli* grow as extracellular or intracellular biofilms inside the cell, on the tissues, or on medical implantation devices (Blankenship & Mitchell, 2006, Anderson et al., 2004, Davies, 2002, Fey & Olson, Foreman & Wormald, Post, 2001). Furthermore, evidence of direct association between chronic persistence and biofilm formation is found in *S. epidermis* through mutation in a single gene that disrupted both phenotypes (Vuong et al., 2004).

The mechanisms of biofilm formation are primarily investigated in genetically tractable species like *B. subtilis*, *Vibrio spp.* and *Pseudomonas spp.* (Kolter & Losick, 1998, O'Toole et al., 1999, Hall-Stoodley & Stoodley, 2002). Despite the distinction in their specific genetic requirements and structural constituents, biofilms of each species are formed through common developmental mechanisms that involve surface attachment, cell-to-cell communication, and synthesis of extracellular matrix (ECM), which encapsulates the resident cells (Kolter & Losick, 1998, Hall-Stoodley & Stoodley, 2002, Hogan & Kolter, 2002,

Chu *et al.*, 2006, Blankenship & Mitchell, 2006, Branda *et al.*, 2005, Danese *et al.*, 2000, Higgins *et al.*, 2007). The constituent microbes in biofilms must reside in, and therefore adapt to, highly complex, heterogeneous and dynamic microenvironments that conceivably could foster phenotypic diversity in the population, a scenario unlikely to be encountered by single-cell planktonic counterparts (Kolter & Losick, 1998). Overall, the encapsulated growth along with phenotypic diversity in the population can be argued as the primary contributors to the extraordinary persistence of biofilms against environmental challenges including antibiotics (Mah & O'Toole, 2001).

The changes in intercellular interactions, cellular physiology and structural compositions associated with development of pathogenic biofilms can also have a profound effect on the outcome of both acute and chronic infections. Accumulation of a set of two quorum sensing signals, CAI-1 and AI-2, in high density cultures of *Vibrio cholerae* negatively co-regulate genes for ECM synthesis as well as virulence (Higgins *et al.*, 2007). This suggests that formation of biofilms and creation of suitable microenvironments in the host through virulence factors are intricately related steps that constitute the colonization phase of an acute infection of *V. cholerae*, and their concomitant down-regulation at high density could possibly be an exit strategy of the pathogen. However, in a chronic infection of *S. aureus* in a mouse model Shirliff and colleagues found that early and late stages of biofilms elicit distinct host responses (Prabhakara *et al.*, 2011). While early stage biofilms triggered a Th1-mediated acute inflammatory response- possibly to create conducive tissue microenvironment for colonization - the old biofilms induced Th2-mediated humoral response that was ineffective on the pathogen - perhaps an immune evasive mechanism that facilitates the chronic survival (Prabhakara *et al.*, 2011).

Taken together, biofilms represent a natural but highly complex life-style of most microbial species, promote persistence of constituent cells in robust structures, and provide unique microenvironments that facilitate extensive phenotypic diversity.

## 5. Could *M. tuberculosis* infections persist as biofilms?

While the long-term persistence of *M. tuberculosis* against the host immune system and antibiotics has striking similarity with the chronic infections of biofilm forming pathogens, it remains unclear if the tubercle bacilli form biofilms in the host. It is, however, noteworthy that *in vitro* cultures of all mycobacterial species grow in complex structures that eventually develop as pellicles on the liquid-air interface, unless a detergent is added as dispersal agent in the medium. Interestingly, such growth pattern of mycobacteria have frequently been noted in the literature as aggregation of cells driven by their surface hydrophobicity, and largely been ignored ever since Dubos and colleagues reported a method to grow dispersed culture of tubercle bacilli without diminishing their virulence (Dubos *et al.*, 1946). However, the emerging concept of microbial persistence in biofilms have recently led several groups to investigate the detergent-free *in vitro* growth of mycobacterial species from the perspective of organized multicellular structures (Hall-Stoodley & Lappin-Scott, 1998, Carter *et al.*, 2003). In one of the first genetic studies of surface associated growth of mycobacteria, Kolter and colleagues observed that an *M. smegmatis* mutant deficient in biosynthesis of acetylated glycopeptidolipid was also unable to attach and grow on an abiotic surface, thus demonstrating a specific genetic requirement for surface-associated mycobacterial growth (Recht & Kolter, 2001). Ojha *et al.* subsequently reported that a

mutation in one of the non-essential chaperone, of *M. smegmatis* specifically retarded the maturation stages of pellicle formation, observed at 4- and 5-day of incubation, without affecting early attachment and growth during first three days of incubation (Ojha *et al.*, 2005). The mutant was also indistinguishable from its wild-type parent in planktonic growth. The maturation defect of the mutant was linked to defective synthesis in mycolic acids as a consequence of the loss of a KasA (enzyme involved in mycolic acid biosynthesis) interaction, which is induced in this phase of wild-type culture (Ojha *et al.*, 2005). The regulated synthesis of mycolic acids was surprising because it is highly abundant in the cell wall, although it is consistent with the subsequent observation of induced synthesis of extracellular free mycolic acids during the maturation stage of the pellicles (Ojha *et al.*, 2010). The free mycolic acids (FM) are released through regulated hydrolysis of mycolyl esters of Trehalose, Trehalose 6',6' dimycolate (TDM), and by cutinase-like serine esterase (Ojha *et al.*, 2010), although other mycolyl esters could also contribute to the FM pool through similar mechanism. One possible candidate could be mycolyl diacyl glycerol (MDAG), a mycolyl ester of glycerol, which is also found in low abundance in impaired biofilms of an *lsr2* mutant of *M. smegmatis* (Chen *et al.*, 2006). The accumulation of FM is likely facilitated through a three-step mechanism: 1) mediated upregulation in *de novo* synthesis of the nascent mycolic acids, 2) processing of nascent mycolic acids into a subset of mycolyl esters through housekeeping mechanisms, and 3) hydrolysis of these mycolyl esters through substrate-specific esterases. The elevated levels of extracellular free mycolic acids during the maturation of *M. smegmatis* pellicles is also consistent with the obvious waxy appearance of the structures, and thus could likely constitute the structure component of the ECM. The significance of *M. smegmatis* growth in pellicles is further highlighted by distinct phenotypes of the bacilli in these structures that are absent in planktonic cell suspension. For example, increased intercellular transfer of genetic materials, and extraordinary tolerance to anti-TB drugs (Ojha *et al.*, 2010, Nguyen *et al.*, 2010). Most importantly, the fundamental properties of mycobacterial pellicles are conserved in *M. tuberculosis* when grown in detergent-free media under defined condition (Ojha *et al.*, 2008). The pathogenic species not only has specific genetic requirement for forming the pellicles (Fig. 2A and B), but also produces large abundance of free mycolic acids in the structure, which expectedly harbor large numbers of drug tolerant bacilli (Ojha *et al.*, 2008) (Fig. 3). Taken together, these *in vitro* studies strongly support the possibility that surface-associated multicellular structures of mycobacteria have all the characteristics of biofilms, developing through distinct growth phases, having specific genetic requirements, and conferring high tolerance to antibiotics. Despite a recent surge in understanding the multicellular structures of mycobacteria, multicellular structures of *M. tuberculosis* in the host have been difficult to define. This in part can be attributed to the conventional image of *M. tuberculosis* as an intracellular pathogen living in phagosome, which is too restrictive for exuberant growth of the pathogen in multicellular structure. However, while *M. tuberculosis* might be restricted to the phagosome in early stages of infection, at later times, especially when lesions contain liquefied caseum and when patients are highly infective, it is likely that many of the bacilli experience an extracellular environment. In a comprehensive histopathological study of TB lesions from 1,500 autopsies, Geroges Canetti documented several lesions including open cavities that had numerous extracellular bacilli growing in multicellular structures (Canetti, 1955). Interestingly, in this 7-year study Canetti microscopically analyzed lesions of various

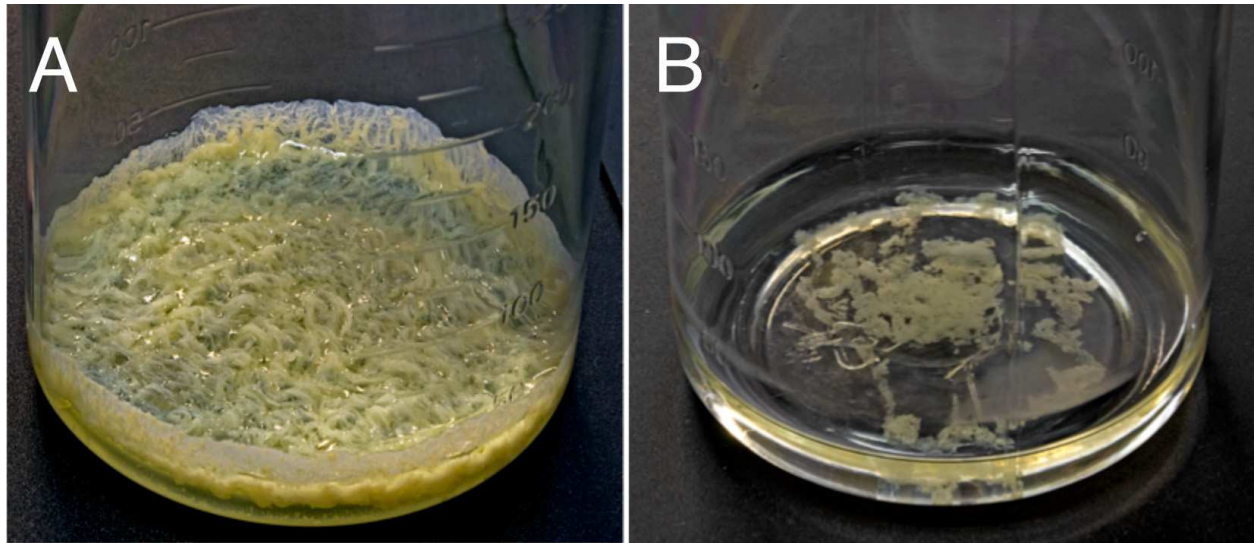


Fig. 2. A. Growth of *M. tuberculosis* biofilms on the liquid-air interface in detergent-free Sauton's medium. B. Insertion of a mariner transposon (Himar) in Rv1013 abolishes the formation of biofilms, although the growth of the mutant in planktonic state remains unaltered (68).

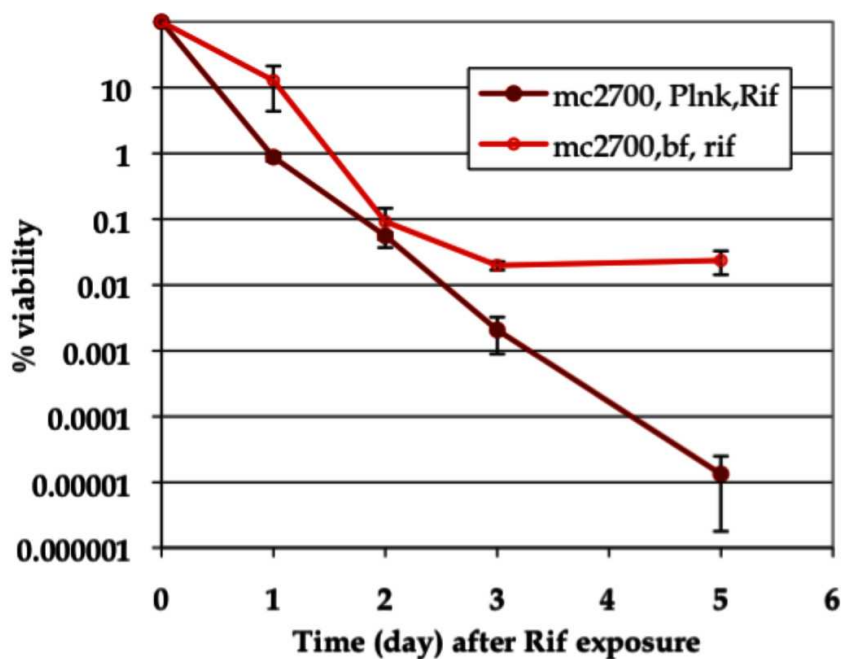


Fig. 3. Presence of rifampicin tolerant persisters is significantly higher in biofilm populations of *M. tuberculosis* than in their planktonic counterparts, as reported by Ojha *et al.* (68).

morphotypes in detail, with equal focus on both the tissue structures as well as bacterial growth (Canetti, 1955). The goal of the undertaking was to bridge the partition between immunopathology and bacteriology of tuberculosis that a student of the disease was always confronted with. This partition ironically continues to be a relevant issue in questions as to how and where bacilli persist in the both latent and active TB. In a recent attempt to locate the bacilli persisting after antibiotic treatment, Orme and colleagues observed the persisters as microcolonies in the acellular rim of granulomas (Lenaerts *et al.*, 2007). Although it was



not clear in their study whether these bacilli were alive or dead, these are reminiscent of the extracellular multicellular structures of bacilli reported in Canetti's study.

## 6. Exploring *M. tuberculosis* biofilms *in vivo*

The *in vitro* studies on mycobacterial persistence in biofilms provide a compelling argument that the extracellular multicellular structures of *M. tuberculosis* in liquefied lesions could be primary foci of persister cells. A basic approach in such a study will involve three critical components: a) imaging *M. tuberculosis* bacilli in intact lesions, b) identifying molecular signatures like free mycolic acids of biofilms in multicellular structures of bacilli, and c) genetically correlating persistence with multicellular structures. Imaging bacilli in intact lesions could be a potentially challenging and expensive approach but is important because conventional processing of tissues with harsh organic chemicals used in typical histopathological protocols likely distort the bacillary architecture. Moreover, the modified cell wall of mycobacteria in biofilms could also render them undetectable by acid-fast staining. Confocal Laser Scanning Microscopy (CLSM) of fluorescently marked *M. tuberculosis* in lesions resected from an animal model that closely mimic human infections, like non-human primates, represents an attractive approach to imaging *M. tuberculosis in vivo* biofilms. The imaging studies could then be followed with detection and analysis of extracellular molecules including lipids and proteins that are associated with multicellular structures. Although the abundance of free mycolic acids in biofilms *in vitro* makes this an excellent candidate, the search should remain open, in case different surface molecules are used for cohesion of the structures *in vivo*. Finally, a systematic *in vivo* analysis of genetically defined mutants that fail to form *in vitro* biofilms could be a powerful strategy for gaining mechanistic insights and identifying drug targets that can dismantle the biofilm structure. It is noteworthy that transposon insertion in *pks16* (Rv1013) and *helY* can impair the development of *M. tuberculosis*, although it is unclear whether the effects of genes products are directly on structural formation or indirectly on adaption of resident bacteria within the structure (Ojha et al., 2008). Mutants in the former category could be especially useful for *in vivo* studies avoiding indirect effects of gene products resulting from changes in the morphologies of the structures.

## 7. Conclusions

Although a short and effective treatment of *M. tuberculosis* infection remains a big challenge to mankind, a solution is unlikely to appear without mechanistic insights into the persistent nature of the pathogen. At the origin of such studies lies a growth model that would reflect the spontaneous behavior of the pathogen. Use of detergents in the process of growing dispersed *in vitro* cultures has arguably misrepresented the physical existence of *M. tuberculosis* in its natural context. In the absence of detergent, the pathogen forms drug tolerant multicellular biofilms, and the complex structures of biofilms undoubtedly hold a treasure of information about the mechanisms that shape their behavior. It is time we focused on these observations to develop new strategies to combat Man's deadliest microbial enemy – *M. tuberculosis*.

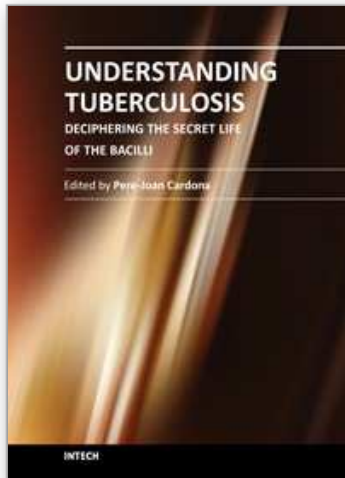
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## **Understanding Tuberculosis - Deciphering the Secret Life of the Bacilli**

Edited by Dr. Pere-Joan Cardona

ISBN 978-953-307-946-2

Hard cover, 334 pages

**Publisher** InTech

**Published online** 17, February, 2012

**Published in print edition** February, 2012

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.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Anil K. Ojha and Graham F. Hatfull (2012). Biofilms of Mycobacterium tuberculosis: New Perspectives of an Old Pathogen, Understanding Tuberculosis - Deciphering the Secret Life of the Bacilli, Dr. Pere-Joan Cardona (Ed.), ISBN: 978-953-307-946-2, InTech, Available from: <http://www.intechopen.com/books/understanding-tuberculosis-deciphering-the-secret-life-of-the-bacilli/biofilms-of-mycobacterium-tuberculosis-a-new-perspective-of-an-old-pathogen>

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