The evolution of *Mycobacterium tuberculosis* dormancy models

Kriti Sikri and Jaya Sivaswami Tyagi*

Department of Biotechnology, All India Institute of Medical Sciences, New Delhi 110 029, India

***Mycobacterium tuberculosis* is a remarkably successful human pathogen owing to its ability to enter into a persistent state. Towards obtaining a better understanding of the mycobacterial persistence mechanisms, several *in vitro*, *ex vivo* and animal models, that mimic one or more features of the host milieu, have been developed. Although none of the models is complete in itself, valuable insights into the persistent state have been derived from each one of them. Here, we review the evolution of the various *in vitro* models for persistent bacterial infection and how the study of dormant bacilli in cell-infection models has provided us with a better understanding of bacterial adaptation and persistence in the face of the challenges posed by the host.**

Keywords: Ascorbic acid, bacterial persistence, DevR, dormancy models, *Mycobacterium tuberculosis*.

Introduction

Bacterial persistence is a hallmark of tuberculosis (TB). The success of *Mycobacterium tuberculosis* as a pathogen is attributed in no small measure to its ability to programme itself into entering prolonged periods of 'dormancy' and resulting in a latent TB infection. It is estimated that approximately one-third of the world's population, namely more than 2 billion people, harbours a latent TB infection. This huge reservoir of infected individuals serves as a never ending source of infection in the community due to the dynamic state of latent versus active infection (Figure 1). The first reports of TB latency appeared eighty years ago when hundreds of sealed cultures of human and bovine isolates that were placed at 37°C were culturable and retained their virulence upon subculture even after 12 years[1]. The authors proposed that the sealed culture bottles resembled healed human lesions where the bacteria enter into dormancy.

Bacterial transmission occurs largely through the aerosol route and lung is the principal organ affected[2]. However, bacterial transmission can also occur in some instances through the oral route by intake of contaminated food, especially milk and milk products[3]. Following the inhalation of aerosols containing *M. tuberculosis*, the bacteria are ingested by alveolar macrophages and also by type-II pneumocytes where they may undergo multiplication, resulting in destruction of the macrophage and infection of neighbouring macrophages that engulf the released bacteria. Tubercle bacilli survive within phagosomes in the macrophages by actively blocking phagosome–lysosome fusion and subsequent killing[4]. Bacterial components, including complex glycolipids and proteins, traffic through the host cell and serve as antigens recognized by the host immune system upon release by exocytosis. Most individuals control the initial infection by mounting a cell-mediated immune response and a localized inflammatory response. At approximately three weeks after the initial infection, bacillary growth and macrophage destruction are balanced. The infected macrophages, through their production of chemokines, recruit inactivated monocytes, macrophages, lymphocytes and neutrophils that aggregate around the infected macrophages which differentiate into multinucleated giant cells, foamy macrophages and epithelioid macrophages. The granuloma evolves into an organized structure in which the macrophage core is surrounded by fibroblasts and lymphocytes which form a fibrous enclosure. Thus the granuloma walls off the mycobacteria and prevents their dissemination. Host cells may also undergo caseous necrotic death which leads to central cavitation and liquefaction within the tuberculous granulomas. The inflammatory reaction is spontaneously contained and in many cases calcified lesions which are particularly common in children, persist for the remainder of the person's life in the absence of active disease[5–9]. Although tubercle bacilli are believed to be unable to replicate within the restricting microenvironment of caseous lesions due to oxygen limitation, acidic environment and the presence of toxic fatty acids, the bacteria may remain in a dormant viable persistent state resulting in a latent infection[10,11]. This infection can persist in an aymptomatic and non-transmissible state throughout a person's life but bacteria serves as a source of long-term infection and may reactivate in later life to cause post–primary tuberculosis in case of lowered immunity, which usually occurs as a consequence of old age, malnutrition or upon immunosuppression, as in HIV-infected patients, and following immunosuppressive therapy[6,12]. This happens when viable tubercle bacteria escape from the granuloma and spread via the lymphatic system and the blood to cause

*For correspondence. (e-mail: jstyagi@aiims.ac.in)

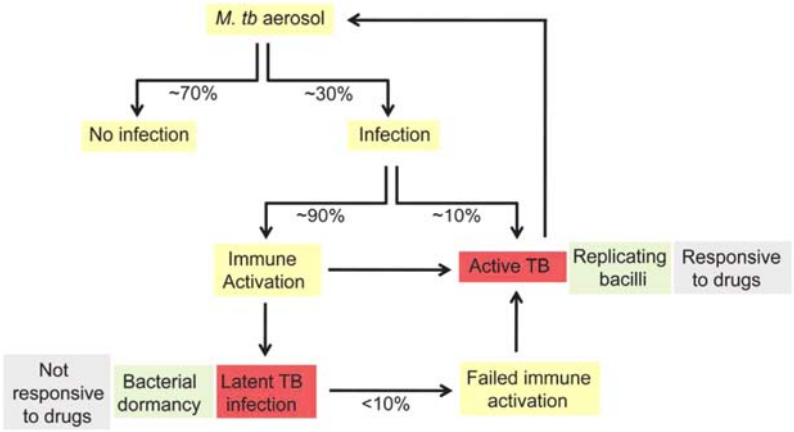

Figure 1. The dynamic state of tuberculosis infection. The usual source of infection is the aerosol generated through cough production by persons with active disease. When inhaled, immune activation occurs in the majority of infected individuals. Encountering the immune defenses, *M. tuberculosis* enters a state of dormancy, leading to a latent infection in a vast majority of the individuals. Only 10% of the infected persons show signs and symptoms of active tuberculosis and are curable with the currently available drugs (drug sensitive TB). The rest 90% of the latently infected individuals act as reservoirs of the bacilli, reactivating in 10% cases in case of immune activation failure and causing active tuberculosis. The reservoir of *M. tuberculosis* aerosol is thus maintained resulting in new infections.

pulmonary TB in the lungs or miliary or extrapulmonary TB.

The challenge of latent TB infection

The latent form of TB is characterized by the absence of clinical symptoms and occurrence of a delayed type hypersensitivity reaction when the individual is challenged with mycobacterial purified protein derivative (PPD)[13]. In macroscopically normal lung tissue without tuberculous lesions from individuals who died of causes other than TB, *M. tuberculosis* DNA was detected in macrophages and also in non-professional phagocytes like type II pneumocytes, endothelial cells, fibroblasts and adipose tissue[14,15]. Long-term intracellular bacterial survival was also reported very recently in human bone marrow-derived $CD271^{+}/CD45^{-}$ mesenchymal stem cells[16]. During latent tuberculosis, tubercle bacilli are believed to considerably reduce or alter their metabolic activity within the phagosome and enter into a state of non-replicating persistence or attain an equilibrium between replication and killing and thereby remain viable for an indefinite period of time. This state of bacterial containment can persist for the lifetime of the individual with a robust immune response. However, the bacteria fail to be contained if the immune status of the host is compromised as mentioned above. Under such circumstances, the centre of the granuloma undergoes caseation and spills viable bacilli into the airways. This leads to the development of a productive cough that facilitates aerosol spread of infectious bacilli[5,17]. In this way, latently infected individuals serve as a seemingly permanent source of the tubercle bacilli in the community (Figure 1). A key challenge in TB control therefore, is to clear the community of latent TB infection and in order to fight latent TB, we need to understand the physiologic and metabolic state of dormant bacilli that reside within the host.

The second major challenge is the lack of drugs effective against latent TB infection. The conventional drugs target pathways such as cell wall synthesis (isoniazid) and RNA synthesis (rifampicin), and have maximum activity against actively replicating bacilli. In order to address this challenge, developing drugs that target dormant bacteria is a priority area in drug development. In recent times, a diarylquinoline drug derivative, commonly known as TMC207, was described, that targeted the ATP synthase of both drug-sensitive and drug-resistant mycobacteria[18]. Importantly, as dormant mycobacteria possess residual ATP synthase activity, TMC207 in nanomolar concentrations, effectively blocked their viability[19]. This drug thus, now christened as Bedaquiline, showed the potential for becoming a good sterilizing agent against *M. tuberculosis* and other mycobacterial species[20] and has been approved recently by the US FDA as a drug for the treatment of MDR-TB. The approval is hailed as a landmark in the history of TB drugs as TMC207 is the first new TB drug to be released for use since the introduction of rifampicin in 1970 (ref. 21).

The past few years have shown a rise in the search for targets of *M. tuberculosis* against which drugs can be developed[22]. Among the proposed targets are isocitrate lyases (Icl1 and Icl2) which are essential for bacterial survival and persistence in both macrophages and mice[23,24]. As these enzymes are absent in human cells, they are proposed as attractive targets for the development of drugs against *M. tuberculosis*. The proteasome complex of *M. tuberculosis* was also proposed as a putative drug target as its inhibition increases the susceptibility of *M. tuberculosis* to reactive nitrogen intermediates produced by infected cells[25]. Besides these, other targets like L-,D-transpeptidase[26,27] and the stringent response

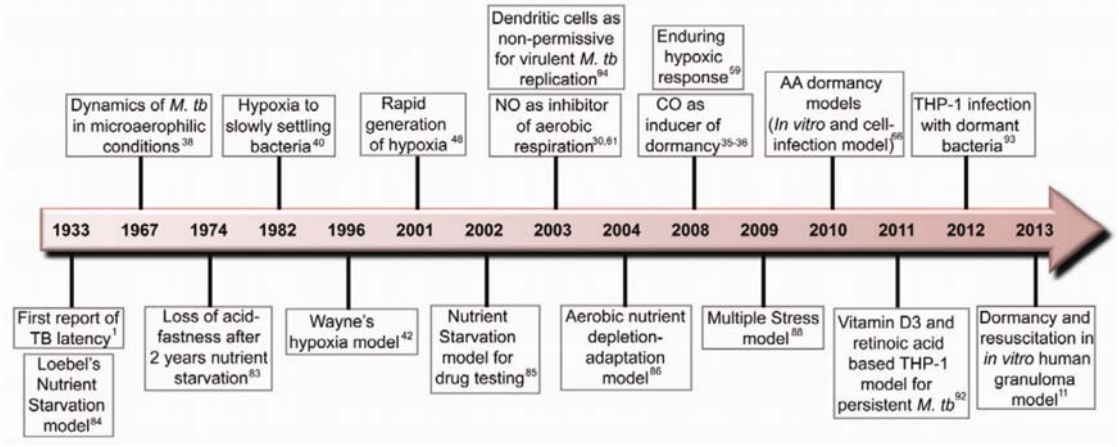

Figure 2. Evolution of *M. tuberculosis* dormancy models. The arrow is not drawn to scale.

protein CarD[28] may also serve as drug targets against dormant *M. tuberculosis* as their deletion limits bacterial persistence. Another potential target is DevR–DevS/DosT (also called as DosR–DosS, see below) that mediates bacterial adaptation to hypoxia, nitric oxide and carbon monoxide[29–36]. Bacteria are believed to be exposed to these gaseous stresses within the granuloma. A phenylcoumarin derivative inhibited DevR activity, blocked hypoxic survival and prevented recovery of *M. tuberculosis* cultures *in vitro*[37]. Thus, this compound showed promise in interfering with the survival of dormant bacteria under hypoxia, and also proved DevR as a promising target for developing molecules against dormant *M. tuberculosis.*

In view of these challenges and the obvious difficulties in studying bacterial dormancy in humans, mycobacterial dormancy model systems assume tremendous importance. The development of *in vitro*, *ex vivo* and animal models has provided us with valuable insights into the adaptation and survival strategies of *M. tuberculosis* in the face of hostile stresses mounted by the host. Here we review the various *in vitro* and cell infection models of dormant *M. tuberculosis* that were developed over the decades (Figure 2).

In vitro models of latency and persistence

It is believed that tubercle bacilli are exposed to multifarious stresses within the host which include hypoxia, nutrient starvation, oxidative and nitrosative stresses, iron limitation and exposure to gaseous stresses such as nitric oxide and carbon monoxide. Accordingly, a number of *in vitro* dormancy models have been developed that attempt to mimic one or more of the putative stresses confronted by the bacterium *in vivo*. The use of these models has provided us with valuable insights into the properties of dormant *M. tuberculosis* as it exists within the host.

Hypoxia model

Hypoxia is the most well-characterized environmental trigger for dormancy. Bacterial autolysis was reported during culture in Sauton's media containing glycerol and was attributed to unbalanced metabolism associated with oxygen limitation[38]. Subsequent pioneering work by Wayne revealed the dynamics of *M. tuberculosis* growth under microaerophilic conditions; the bacilli were observed to grow in an arithmetic manner as opposed to the logarithmic mode in aerobic cultures[39]. This further led to the development of the first *in vitro* model for dormancy based on hypoxia. In this model, settling tubercle bacilli in undisturbed liquid cultures are exposed to self-generated oxygen depletion and undergo an orderly process of metabolic shutdown[40]. The dormant bacteria develop resistance to rifampicin and isoniazid, but are sensitive to the anaerobic bactericide metronidazole[41]. Though the model provided a good *in vitro* system for studying the overall dormancy response of *M. tuberculosis*, the heterogeneous population formed by the settling bacteria did not provide an opportunity to study the discrete events in the adaptation of the bacilli to dormancy. Refinements were made in the model, wherein *M. tuberculosis* cultures were exposed to limited headspace volumes of air in stirred sealed tubes agitated in a controlled way which led to gradual depletion of all the available oxygen during bacterial growth. In this model, popularly referred to as the Wayne model, the bacteria progress through two stages of non-replicating persistence[42]. The first stage termed as NRP-1 (non-replicating persistence stage 1) is characterized by a cessation of cell division, and DNA, RNA and protein synthesis, followed by a more widespread 'shutdown' of metabolism during the second stage known as NRP-2. The transition into NRP-1 occurs when the exponentially growing cells have consumed oxygen to a level of 1% saturation, whereas NRP-2 was reached when oxygen levels are reduced to less than 0.06% saturation. A gradual depletion of oxygen leads to a non-replicating persistent state characterized by bacteriostasis and metabolic, chromosomal and structural changes of the dormant bacteria[42]. Upregulation of two enzyme systems, glycine dehydrogenase, that leads to reductive amination of glyoxylate to glycine[40] and nitrate

reductase, that results in the reduction of nitrate to nitrite[43], have been suggested to represent potential alternative energy sources for NRP tubercle bacilli[44]. Under conditions of hypoxia, the bacilli are known to downregulate key components of the electron transport chain and also maintain lower ATP levels[45,46]. But sustained production of ATP, regeneration of NAD and maintenance of proton motive force are required in hypoxic conditions also. The upregulation of the anaerobic type-II NADH dehydrogenase and the energetically more efficient cytochrome bd oxidase were proposed as compensatory mechanisms during hypoxia[13].

Variations of the Wayne model include the standing of aerobic cultures in tubes and plates[47], the rapid generation of hypoxia[48], culturing bacteria in vented cap tissue culture flasks in an incubator where low oxygen tension was maintained[49]. These models have contributed substantially to our understanding of the molecular mechanisms underlying the 'dormancy response'[31,50].

The relevance of these hypoxia-based models was established by the demonstration of hypoxia within pulmonary lesions. The partial pressure of atmospheric oxygen is ~150–160 mmHg and it drops to ~59 mmHg in healthy lungs[51]. Direct measurements of oxygen tension within pulmonary lesions of guinea pigs, rabbits and non-human primates using pimonidazole hydrochloride revealed the lesions to be severely hypoxic[52] and intraphagosomal oxygen concentrations were shown to be only ~1.9 mmHg[51,53].

Proteomic analysis of *M. bovis* BCG bacteria in the Wayne model of hypoxia revealed the upregulation of several proteins including DevR and HspX[54]. The severe loss of viability of a *M. bovis* BCG mutant strain lacking DevR indicated its essential role in bacterial adaptation to hypoxia[54]. Active efforts of numerous research groups led to a characterization of the DevR/DosR response regulator and its adaptive functions. The exposure of *M. tuberculosis* to hypoxic conditions results in a rapid induction in a DevR-dependent manner of approximately 48 genes that are referred to as DevR/DosR regulon[29]. These genes are expressed from approximately 22 promoters that have two or more DevR binding sites composed of 18–20 bp palindromic sequence[29,49,55]. DevR function is also essential for the adaptation of *M. tuberculosis* in a gradual hypoxia development model[56,57]. Upregulation of the DevR dormancy regulon was noted during early NRP with the expression of some of the genes returning to baseline during late NRP[58]. In a different model where hypoxia is generated very rapidly, transient induction of the DevR regulon was followed by induction of a set of 230 other genes named as the enduring hypoxic response (EHR) when a subset of DevR regulon genes returned to the baseline after 24 hours[59]. Also, genes involved in mycolic acid modifications were induced during adaptation to NRP but repressed thereafter, suggesting that the bacilli were modifying the degree of saturation of cell wall mycolic acids in order to prepare for the ensuing non-proliferative phase[58].

Nitric oxide (NO), at concentrations ≥1 mM, also acts as a potent inhibitor of bacterial aerobic respiration[60] and also strongly induced the dormancy regulon via DevR[30,61]. The overlap in responses mediated by NO and by hypoxia indicates that these apparently quite different treatments share a common mechanism of action[61]. Voskuil and colleagues[30] proposed a model to describe the relationship between the tissue concentrations of NO and O_2. According to this model, NO may compete with oxygen to inhibit respiration and activate DevR, which in turn switches on the dormancy regulon[30]. Subsequently, both DevS and DosT (Rv2027c), an orphan sensor kinase, were shown to be heme sensors[62–64]. Both the sensors were shown to avidly bind to NO resulting in the activation of their kinase function and aerobic induction of DevR regulon gene expression[63].

Thus, hypoxia, NO and CO represent gaseous stresses which may regulate gene expression in overlapping pathways ultimately leading to a cessation of bacterial growth. The bacilli respond to a decrease in respiration within granulomas by initiating a transcriptional response that stabilizes vital cellular components and enables survival during extended periods of latency. The selective induction of DevR genes by CO, NO and hypoxia provides compelling evidence that *M. tuberculosis* encounters one or more of these conditions in the murine lung during the course of infection. This hypothesis is supported by the observation that most *M. tuberculosis* genes in the NO/dormancy/hypoxia gene set are strongly induced within INF γ-activated bone marrow-derived murine macrophages from wild-type mice, but not in activated macrophages from inducible nitric oxide synthase knockout mice[65]. Thus, in the intra-phagosomal environment of IFN γ-activated mouse macrophages, the induction of these genes is likely to be NO-dependent. An important function of NO production and granuloma formation by the immune system is to limit aerobic respiration and impair growth of *M. tuberculosis*, an obligate aerobe. Reprogramming of the transcriptional response enables bacterial survival during extended periods of latency. This response occurs at nontoxic concentrations of NO, suggesting that NO serves as a host environmental signal that discloses the level of immune activation to the host[30].

A serendipitous discovery was made in our laboratory that the DevR dormancy regulon was induced by vitamin C. This activation was ascribed to the rapid development of hypoxia owing to the O_2 scavenging properties of vitamin C. It was also demonstrated that vitamin C induces bacteriostasis and an INH-tolerant phenotype both in *in vitro* cultures and in the THP-1 cell infection model. Phenotypic tolerance to INH is generally accepted to be an indicator of physiological adaptation similar to that observed under conditions of gradual oxygen depletion. INH tolerance is not attributed to mutations at the genetic

level as bacteria resume aerobic growth in vitamin C-free media (K. Sikri, unpublished data). Genetic analysis of DevS and DosT sensor kinase mutants provided insights into the relative roles of DevS and DosT during *M. tuberculosis* adaptation in the 'AA-dormancy infection model'[66]. It was suggested that rapid hypoxia development by vitamin C enables an initial DosT response and gradual O_2 depletion majorly supports signalling via DevS sensor and an amplified genetic response. Vitamin C was also found to induce the expression of acid-responsive and oxidative stress response genes in *M. tuberculosis*, for example, *icl* (encoding isocitrate lyase, a key enzyme of the glyoxylate cycle), *mymA* operon genes (having a role in mycolic acid synthesis), *ahpC* and *katG* (that provide protection against reactive oxygen and nitrogen intermediates)[66]. However, another report demonstrated that DevS, but not DosT, induced DevR on addition of ascorbate, a cytochrome C reductant, under aerobic conditions. On this basis, the authors argued the role of electron transport system in DevS control of DevR regulon and supporting the role of DevS as a redox sensor[67].

Currently, two theories have been proposed for the biochemical properties of DevS and DosT kinases. One states that both DevS and DosT directly bind to oxygen resulting in a kinase 'off' state. When oxygen becomes limiting and owing to the differential affinity of the two kinases for oxygen, DosT kinase activity is turned on by the release of bound oxygen resulting in phophotransfer relay to DevR[62,63,68,69] and downstream activation of the regulon[66]. Others are in agreement with DosT being an oxygen sensor but state that DevS is a redox sensor based on the *in vitro* oxidation rate and the stability of the oxy complex[64,70]. As aerobic conditions maintain DevS in the inactive oxidized/oxy form and DevR induction via DevS occurs in response to reduced electron transport system, it was suggested that both ideas may be correct; DevS may be acting as a oxygen sensor as an 'off switch' and a redox sensor as an 'on switch'[67].

Evidence for the *in vivo* relevance of DevR regulon dormancy antigens comes from the detection of immune responses directed towards these antigens, in individuals harbouring a latent TB infection[71,72]. For example, immune responsiveness to HspX was shown to be more predominant in latently infected individuals[73]. Furthermore, cellular response to another DevR regulon antigen encoded by Rv2628 was found to be associated with cured TB cases[74]. The detection of these responses provides support for the *in vivo* expression of these antigens and has the potential to be utilized for the diagnosis of latent infection.

There is some confusion in the literature regarding the nomenclature of *devR* which is also referred to as *dosR*. In 1998, *Rv3133c* and *Rv3132c* were assigned as one among 11 two-component systems in the annotated *M. tuberculosis* H37Rv genome[75]. At that time, with the exception of *mtrA–mtrB*[76], the others were designated as putative two-component systems. The *dev* genes of *M. tuberculosis* were identified in 1993 by Kinger and Tyagi on the basis of their differential expression in the virulent H37Rv strain versus the avirulent H37Ra strain[77] and in 2000, two of the *dev* genes were designated as *devR* and *devS* based on sequence similarity to two-component systems[78]. Accordingly, *Rv3133c* and *Rv3132c* were annotated as *devR* and *devS* respectively, in the TubercuList database (http://genolist.pasteur.fr/TubercuList/). Expression analysis established that *devR–devS* constitute an operon along with an upstream gene *Rv3134c*. DevR protein expression was also demonstrated in *M. tuberculosis* cultures and within infected monocytes[78]. In 2002, recombinant DevS protein was shown to be biochemically active in an autophosphorylation reaction characteristic of histidine kinases[79]. In 2002, based on proteomic and genetic analysis of *M. bovis* BCG, the *Rv3133c* homologue in *M. bovis* BCG was named as *dosR*[54] and this name is also used sometimes in the literature to refer to *devR* of *M. tuberculosis*. Subsequently, in 2008, when *devR* expression was shown to be downregulated in H37Ra with respect to H37Rv probably as a consequence of a Ser219Leu mutation in PhoP from H37Ra, a link between PhoP and DevR was suggested and a possible explanation was proposed for the differential expression of *devR* in H37Ra[80].

Nutrient starvation model

M. tuberculosis is believed to reside in environments where nutrients and other essential cofactors are limiting[10,81,82]. *M. tuberculosis* cultures starved in distilled water displayed altered morphology and staining properties in comparison to actively growing aerobic cultures; after a prolonged starvation period of two years, the bacteria regained their acid-fastness upon transfer to a nutrient-rich medium[83]. As early as 1933, investigators of *M. tuberculosis* metabolism established that the bacteria remained viable in nutrient deprived condition (phosphate buffer saline) and respiration occurred at minimal levels, indicating low/inactive metabolism in the surviving bacteria[84].

Based on these findings a nutrient starvation model (also referred to as the Loebel model) was employed to characterize the adaptive mechanisms of *M. tuberculosis* in response to nutrient limitation[85]. In this model, nutrient starvation is initiated by the transfer of *M. tuberculosis* cultures grown in a nutrient-rich medium to a nutrient-limiting medium such as phosphate-buffered saline and prolonged incubation under these conditions in the presence of oxygen. *M. tuberculosis* cultures grown in this fashion exhibit a 'dormant' phenotype in that they are bacteriostatic and show no change in viability over a 6-week incubation period. They develop phenotypic tolerance to the anti-tubercular drugs, INH and rifampicin, as well as to metronidazole, thus showing that the nutrient starved bacilli are in a dormant state. Unusual colony

morphology and staining property of these cultures, which is reversed following transfer back to the nutrient-rich medium, as well their reduced respiration rates emphasize their dormant nature. At the genetic level, nutrient-starved cultures exhibited a global downshift in gene expression. Genes involved in transcription, amino acid biosynthesis, biosynthesis of cofactors, prosthetic groups and carriers, DNA replication, repair, recombination and restriction/modification, energy metabolism, lipid biosynthesis, translational and post-translational modification, and virulence, were all expressed at lower levels, whereas genes involved in stringent response and long-term survival were induced. Furthermore, two dimensional gel analyses of cell extracts confirmed that many alterations noted by gene expression result in reduced protein levels.

An aerobic nutrient depletion–adaptation model was introduced by Hampshire *et al.*[86], that allowed the maintenance of long-term stationary phase culture in a fermenter vessel, by controlling parameters like pH, temperature and dissolved oxygen content. This model was based on the hypothesis that a sub-population of *M. tuberculosis* that survives *in vitro* during the stationary phase may represent the population of the bacilli that persist during infection. The *M. tuberculosis* culture was grown with a constant dissolved oxygen content of 50% for 100 days and nutrients were allowed to exhaust. The adaptive response to the depletion of glycerol and glucose was observed up to 75 days. Gene expression microarrays analysis over the entire period of culture indicated that the main energy source during this 75-day period was through the β-oxidation of fatty acids. Reactivation occurred after 75 days, suggesting that the surviving population was well adapted to start dividing again. During this reactivation phase, gene expression patterns were also found to change in support of the events of DNA repair and chromosome division.

The nutrient starvation 'Loebel' model[84] and the hypoxia 'Wayne' model[42] have both been used to characterize the adaptation of *M. tuberculosis* during survival for extended periods upon exposure to environmental stresses. While the 'Loebel' model is based on exposing bacteria to nutritional stress in the presence of oxygen, the 'Wayne' model is based on bacterial exposure to hypoxia in nutritionally replete medium. Not surprisingly, Loebel and Wayne bacilli are in metabolically different states which suggest that the bacteria using different adaptive mechanisms to attain the common end goal of dormancy. This diversity in adaptation response is exemplified by the finding that a phenylcoumarin inhibitor of DevR attenuates bacterial adaptation to hypoxia but has no effect on dormancy development under nutrient starvation[37]. It was also noted that the ATPase inhibitor TMC207 compromises hypoxic survival but does not affect the viability of 'Loebel' bacteria to a significant extent[87]. Nutritionally starved 'Loebel' bacilli demonstrated a higher level of drug tolerance and decreased sensitivity to ATP depletion as compared to 'Wayne' bacilli. Nutrient starved bacilli continue to use oxygen and nitrate can be utilized as an external electron acceptor, which does not appear to be the requirement for hypoxic bacilli. However, a common theme in both models is the requirement for lowering of ATP levels and maintaining ATP homeostasis for non-replicating survival and viability[87].

Multiple stress models

As mentioned above, *M. tuberculosis* transitions into a dormant state and acquire drug resistance upon exposure to host-derived stresses during infection. An *in vitro* multiple stress model was developed to more closely mimic the scenario that *M. tuberculosis* encounters within the host. Such a model was expected to disclose the metabolic activity of a bacterial population more akin to intracellular dormant bacteria[88]. This model exposed bacteria to the combined stresses of low oxygen (5%), high CO_2 (10%), low carbon and nitrogen nutrients and acidic pH (5.0). In response to these stresses, *M. tuberculosis* stopped replicating, lost acid-fastness, became tolerant to anti-tubercular agents like rifampicin and INH and accumulated wax esters and triglycerides. The genes involved in the biosynthesis of these lipids were found to be upregulated. Among the 15 triglyceride synthesis genes, *tgs1* (*Rv3130c*), a member of the DevR dormancy regulon, was found to be maximally induced. A *tgs1* mutant defective in accumulating storage lipids was found to be sensitive to rifampicin and INH and on this basis the authors proposed a link between storage lipid accumulation and antibiotic tolerance[88]. In addition to *tgs1*, *tgs2* (*Rv3734c*) and *tgs4* (*Rv3088*) were also upregulated. Triacylglycerol synthesized under hypoxic conditions has also been suggested to serve as a source of energy during dormancy and for resuscitation of *M. bovis* BCG[89]. These genes were previously shown to be induced under nutrient starvation[85] and acidic stresses suggesting the multi stress nature of this model[90].

Cell infection models of *M. tuberculosis* dormancy

Although *in vitro* models have provided valuable insights into the 'dormancy' response of *M. tuberculosis*, they have limitations of not examining bacterial adaptation in the intracellular milieu. *M. tuberculosis* is an intracellular pathogen and obviously models incorporating 'dormant' bacterial analysis in an intracellular context become highly relevant. Such models bypass the limitations of axenic cultures on one hand and the complexities of animal models on the other hand.

Presently, much of the information obtained about *M. tuberculosis* infection has come from *ex-vivo* models where *M. tuberculosis* undergoes replication[91]. However,

these models were not designed to study specifically intracellular 'dormant organisms'. The 'vitamin C or ascorbic acid (AA)-dormancy infection model' overcomes the limitations of existing intracellular models[66]. In this model, THP-1 cells are exposed to vitamin C post-infection with *M. tuberculosis.* A rapid induction of the DevR dormancy regulon occurred on exposure to vitamin C, indicating that the intracellular environment is hypoxic. Moreover, a significantly greater fraction of the bacteria from vitamin C-treated cultures acquired an INH-tolerant phenotype (~35%), in comparison to bacteria in control THP-1 infected cells (~1%). As the transfer of vitamin C-exposed cultures to vitamin-free media restored growth properties and INH sensitivity (unpublished results), this model offers an alternative to other models of non-replicating persistence of *M. tuberculosis* and may be used to study host–*M. tuberculosis* interactions during dormancy and also during reactivation.

Vitamin D3 and retinoic acid were later used for the development of a THP-1 based cell infection model of *M. tuberculosis.* The authors reported that *M. tuberculosis* persisted for 60 days within multinucleated giant cells formed on differentiation of THP-1 with vitamin D3 and retinoic acid, without the formation of colonies on culture plates[92].

Another model has recently been described, where THP-1 cells are infected with non-replicating NRP-2 tubercle bacilli generated using the Wayne model of dormancy[93]. In comparison to aerobic *M. tuberculosis*, dormant bacteria were better contained within phagosomes. Moreover, the phagosomes infected with aerobic bacilli were lysed as bacteria start replicating, while those harbouring dormant *M. tuberculosis* remained stable. The authors studied the expression of selective *M. tuberculosis* and THP-1 genes. They found that the dormancy-related genes of *M. tuberculosis* (*hspX*, *devR*) and stress-induced sigma factors (*sigB* and *sigE*) were upregulated whereas *esat-6*, a gene more typical of the replicating form, was downregulated. Dormant *M. tuberculosis* induced host *cox2* gene expression along with the secretion of PGE2, to counteract necrosis induced by the bacteria. They thus described a model which can help understand how dormant bacilli survive intracellularly and influence the maintenance of hypoxic granuloma.

Yet another model uses dendritic cells (DCs) that have been shown to be non-permissive for the growth of virulent *M. tuberculosis*, unlike monocytes/macrophages where the bacilli replicate. Mycobacterial persistence in DCs, which mature as a consequence of the infection, maybe attributed to reduced communication with host cell recycling and biosynthetic pathways. This is thought to impair access of intracellular bacilli to extracellular nutrients like iron and cholesterol, thus allowing bacteria to enter into a state of dormancy[94]. When the transcriptional responses of *M. tuberculosis* within macrophages and DCs were compared there was a greater induction of stress-responsive genes in DCs. Mycobacterial stress response signatures specific for DCs were identified. The *ald* gene encoding alanine dehydrogenase was upregulated in DCs, suggesting that under low oxygen conditions within DCs, alanine dehydrogenase contribute to the regeneration of NAD^+. Many of such genes (including members of the DevR regulon and *cydA-D* involved in respiration during limiting oxygen conditions) known to be involved in bacterial adaptation to low oxygen conditions, were found to be over expressed in DCs. Several of the genes involved in nutrient limiting conditions (e.g. *glnH*, *pdhA/B*, *cysD*, cholesterol metabolism gene cluster, etc.) were also found to be upregulated in DCs. The authors of the study thus demonstrated at the genetic level that DCs may serve as a model for *M. tuberculosis* persistence[95].

Genes constituting the toxin–antitoxin (TA) systems have also been found to be upregulated in persisters[96]. *M. tuberculosis* has a large number of TA loci, including those belonging to the *rel* and VapBC family, which were shown to be capable of inducing bacteriostasis and degrading mRNA in *M. smegmatis*[97,98]. The changes in the TA concentrations during stress conditions may lead to a metabolic shutdown and assist in *M. tuberculosis* dormancy[96]. Members of the *rel* family (*relE*, *relF* and *relK*) have also been shown to be upregulated in *M. tuberculosis* within macrophages[99].

Most recently, an *in vitro* human granuloma model for dormancy and resuscitation of *M. tuberculosis* upon immune suppression has been described[11]. The authors demonstrated the formation of granuloma containing multinucleated giant cells in response to infection with *M. tuberculosis*. Within the *in vitro* granuloma, the bacilli were shown to enter a state of dormancy as demonstrated by the loss of acid fastness and the accumulation of lipid bodies, as well as tolerance to rifampicin. The bacteria resuscitated on treatment with anti-TNFα antibody, as TNFα is known to maintain *M. tuberculosis* in the dormant state. The state of dormancy was also evident from the upregulation of dormancy markers such as *tgs1*, *gltA1*, *citA*, *icl*, *hspX* and *devR* (*dosR*). The treatment with anti-TNFα antibody resulted in a downregulation of these genes and upregulation of the genes encoding resuscitation promoting factors (*rpfA*, *rpfB* and *rpfC*), genes involved in energy generation (*nuoA*, *nuoB*, *nuoE*, *atpA* and *atpB*) and transcription (*rpoA* and *rpoB*), thus indicating reactivation of the bacilli from a state of dormancy. The product of gene *tgs1* is known to be involved in lipid accumulation during stresses that lead to dormancy and the *lipY* gene product is known to mobilize stored triglycerides during reactivation. Mutations in these genes impaired the ability of the bacteria to enter dormancy and reactivate respectively. This model is the first of its kind that can be used to nearly accurately mimic and study the *in vivo* situation of dormancy and reactivation of the tubercle bacillus.

Concluding remarks

M. tuberculosis dormancy models have provided valuable insights into the mechanisms utilized by *M. tuberculosis* during their adaptation to a dormant lifestyle. These models have been available in various forms from as early as 1933. The most widely studied and characterized bacterial stress response has been to hypoxia and many versions of the model that differ in the way hypoxia is created have further contributed to our understanding of the bacterial adaptation response to hypoxia. All these models identified DevR as the major dormancy regulator in case of hypoxia. The nutrient starvation model mimics another condition that prevails within granuloma where the supply of nutrients is limiting. This model provides insights into nutrient utilization strategies employed by bacteria within the granuloma. Besides hypoxia and nutrient starvation, bacteria also encounter pH and oxidative stresses within the host and it becomes relevant to understand bacterial adaptation to the combined effect of these stresses. In this regard the multiple stress model[88] and the vitamin C-based model[66] are expected to provide an integrated view of bacterial adaptation during dormancy induced by multiple stresses upon infection. Furthermore, the cell-infection models provide an opportunity to delineate the cross talk between host and dormant *M. tuberculosis*. It is now feasible, as never before, to capture a holistic view of this adaptive process using genome wide analysis tools of transcriptomics, proteomics and metabolomics. Efforts are underway in various laboratories to refine the existing models and derive a deeper understanding of the pathogenic processes that contribute to *M. tuberculosis* adaptation to a dormant lifestyle.

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