Mycobacterium aurum is Unable to Survive *Mycobacterium tuberculosis* Latency Associated Stress Conditions: Implications as Non-suitable Model Organism

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Abstract *Mycobacterium tuberculosis* manages to remain latent in the human body regardless of extensive chemotherapy. Complete eradication of tuberculosis (TB) requires treatment strategies targeted against latent form of infection, in addition to the current regimen of antimycobacterials. Many in vitro and in vivo models have been proposed to imitate latent TB infection, yet none of them is able to completely mimic latent infection state of *M. tuberculosis*. Highly infectious nature of the pathogen requiring BSL3 facilities and its long generation time further add to complications. *M. aurum* has been proposed as an important model organism for high throughput screening of drugs and exhibits high genomic similarity with that of *M. tuberculosis*. Thus, the present study was undertaken to explore if *M. aurum* could be used as a surrogate organism for studies related to *M. tuberculosis* latent infection. *M. aurum* was subjected to in vitro conditions of oxygen depletion, lack of nutrients and acidic stress encountered by latent *M. tuberculosis* bacteria. CFU count of *M. aurum* cells along with any change in cell shape and size was recorded at regular intervals during the stress conditions. *M. aurum* cells were unable to survive for extended periods under all three conditions used in the

study. Thus, our studies suggest that *M. aurum* is not a suitable organism to mimic *M. tuberculosis* persistent infection under in vitro conditions, and further studies are required on different species for the establishment of a fast growing species as a suitable model for *M. tuberculosis* persistent infection.

Keywords *M. tuberculosis* · Latent · Persistence · *Mycobacterium aurum* · Acidic stress · Non-replicating persistence

Introduction

Tuberculosis (TB) achieves its success in being the largest bacterial killer around the globe with an annual incidence of 8.8 million cases and 1.4 million deaths every year. TB infection can be categorized into active and latent forms. Active TB involves population displaying characteristic symptoms of the disease and harboring proliferating *Mycobacterium tuberculosis* bacteria. Latent infection is characterized by an asymptomatic state of the population, acting as carrier of the infection. The current regimen of antimycobacterials is effective against active TB, but is largely ineffective in eliminating the *M. tuberculosis* latent infection [1, 2], emphasizing the need for further studies in this direction.

One-third of the human population is predicted to carry *M. tuberculosis* latent infection [3, 4]. Studies related to the latent or dormant form of *M. tuberculosis* bacilli are largely hampered by our limited understanding of the bacilli under latent or dormant stage. Thus, latent tuberculosis infection poses major chokepoint in worldwide tuberculosis control efforts. Strategies are required for identification of novel drug and vaccine targets specific to latent form of *M.*

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tuberculosis infection, thereby fostering the development of effective and potent sterilizing drugs against latent TB. Inclusion of drugs targeted at latent bacilli to the existing anti-tubercular arsenal would help in efficient and shorter therapeutic regimen against the etiological agent, leading to effective cure and complete eradication of *M. tuberculosis*.

As processes involving latency and reactivation take a long time to manifest in humans, various in vitro and in vivo models have been proposed to study latent infection. Owing to long generation time and highly infectious nature of *M. tuberculosis*, several fast growing mycobacterial spp. have been used as surrogates for rapid screening of drugs, and as models of latent infection. *M. smegmatis* remains most widely used organism for rapid investigation of genes under *M. tuberculosis* associated in vitro latency conditions [5, 6] as well as for drug screening. However, *M. marinum* [7, 8] and *M. avium* [9] have also been used as surrogates for the same, yet none of them is completely able to mimic *M. tuberculosis* human latent infection under in vitro conditions.

Till date, no holistic model for latent *M. tuberculosis* infection is available that completely recapitulates conditions of human latent tuberculosis infection (LTBI). Thus, it is imperative to explore and establish new and better model organisms of latent tuberculosis for devising effective control strategies against latent infection. *M. aurum* has a generation time of 2.5 h and easy to handle due to its non-infectious nature. It has the ability to survive in macrophages and can be easily identified from contaminants due to an orange pigmentation. *M. aurum* also possesses a cell wall profile similar to that of *M. tuberculosis* and drug resistance patterns, and shares intracellular therapeutic targets and gene-organization [10]. These merits motivated us to consider and confirm if *M. aurum* could be used as surrogate organism for *M. tuberculosis* latency model.

Granuloma comprises of a thick wall of various immune cells surrounding the bacilli that are embedded in the central core of necrotic tissue. These granulomas cordon the bacilli from dissemination into the body and hence control the infection. Inside such granulomas, bacilli are exposed to various stress conditions, which form the basis of existing in vitro models of latent TB infection. Thus, to study the behavior of *M. aurum* under stress conditions associated with *M. tuberculosis* latency, *M. aurum* cultures were subjected to conditions of hypoxia, nutrition starvation, and acidic stress (conditions present inside granuloma), with an underlying expectation that it could be exploited as a surrogate organism to study *M. tuberculosis* latent infection.

Materials and Methods

Bacterial Strains and Growth Conditions

All experiments were carried on *Mycobacterium aurum* A+ (received as kind gift from CDRI, Lucknow), which was grown in Luria–Bertani (Hi-Media) or MB7H9 (Difco) broth, supplemented with 0.5 % glycerol (Fischer Scientific) and 0.2 % Tween80 (Bio Basic Inc), and plated on Nutrient agar (Hi-Media) supplemented with 0.05 % Tween80 [NAT]. All cultures were grown at 37 °C with constant shaking at 200 rpm to obtain actively growing aerobic cultures. The purity of the cultures as well as changes in morphology as an attribute of bacilli under persistent form was checked by Ziehl–Neelsen (ZN) staining following standard protocols. Any deviation from normal colony morphology and pigmentation characteristic of *M. aurum* was also recorded at each time point during the study. Keeping in view the clumping nature of the genus, cultures were thoroughly vortexed prior to CFU determination in all the studies.

Cell Viability Under Hypoxia Induced Stress Conditions

Hypoxia induced NRP (Non-replicating Persistence) model described by Dick et al. [11] was followed for determination of cell viability and study of growth kinetics of *M. aurum* under hypoxic conditions. Cells were grown to an optical density of 0.4–0.7 (A_{600} [12, 13]), and methylene blue was added as an indicator of oxygen concentration [14, 15] to a final concentration of 1.5 µg/mL and mixed. 7.5 mL of this actively growing culture was dispensed into 15 mL glass vials, thus maintaining head space ratios (HSRs) of 0.5. Vials were capped with rubber septa, sealed with vacuum grease and incubated at 37 °C for 45 days. Continuous shaking was avoided to maintain gradual oxygen diffusion from air space to medium. Oxygen depletion was monitored by decolorization of methylene blue. The viability of the culture was checked by CFU counts at regular intervals. Also changes in staining characteristics and deviation from colony morphology and pigmentation were also determined at each time point. CFU counts of the actively growing culture grown to an optical density of 0.4–0.7 (A_{600}), was compared with an aliquot of the same culture containing methylene blue (1.5 µg/mL) to study the effect of methylene blue on *M. aurum* growth and viability. Growth of the cultures with and without methylene blue was followed till stationary phase.

Cell Viability Under Nutrition Depletion Induced Stress Conditions

With an aim to achieve NRP state of cells, *M. aurum* was subjected to nutrient-starvation stress conditions [1, 16], with minor modifications. Briefly, exponentially growing bacilli at an optical density of 0.5–0.9 (A_{600}) were harvested by centrifugation (2500g, 10 min), and washed twice with PBST [PBS supplemented with 0.05 % Tween80] to remove any media components. Finally, cells were suspended in the same volume of PBST as that of culture media in which they were grown. Cells were starved without media (carbon source) for 30 days. CFU counts of the bacteria were done at regular intervals to check for viability and any deviation in the cell number of the culture. Changes in ZN staining characteristics, colony morphology and pigmentation of *M. aurum* cells were also observed at each time point during the course of the study.

Cell Viability Under Acid Induced Stress Conditions

Mycobacterium aurum was subjected to acid induced stress conditions, as acidic environment is reported to exist inside granuloma, the seat of latent bacilli. For study of growth kinetics under acid induced stress condition, *M. aurum* culture was overgrown in MB7H9 media (pH 6.5) for 3–4 days till late exponential phase [optical density 1.8–2.0 (A_{600})]. Cells were pre-adapted (by overgrowth) before subjecting them to stress conditions, as pre-adaptation is considered to be an important feature for survival under acid induced stress [17]. Stationary phase cells were washed twice with PBST to remove any traces of exhausted media. Cells were then resuspended in separate flasks of MB7H9 media at pH of 5.5 or 4.5, along with flasks containing MB7H9 media at pH 6.5 as control. Viability of the cells was checked by determination of colony forming units (CFU) at regular intervals till 4 weeks post transfer to fresh media. Changes in staining properties and any deviation from normal colony morphology and pigmentation characteristic were also determined at each time point during the study.

Statistical Analysis

Data shown comprises of mean of readings taken from three independent experiments with standard deviation in error bars. CFU was determined in duplicate at each time point.

Results

Mycobacterium aurum Cells Were Unable to Survive Under Hypoxia Induced Stress

Mycobacterium aurum cells failed to survive for extended periods of time when subjected to hypoxia induced stress conditions (Fig. 1a). Sealed vials containing *M. aurum* cultures showed fainting of methylene blue color after day 6, and complete discoloration was observed between days 10 and 12 indicating hypoxic environment. *M. aurum* cells showed viability up to day 16 under gradual oxygen depletion conditions. A sharp decline in CFU count was observed 16 days post sealing of the vials and no CFU count was observed after 20 days. Cell viability was further checked till 45 days post sealing of vials with no reversal of cell viability (data shown only till 30 days post sealing and 20 days after hypoxia). *M. aurum* cells displayed loss of acid fastness and shortening of bacilli after 1 week of sealing till the cells showed viability [Supplementary Fig. S1(b)]. During the complete study, viable bacilli maintained orange colony color. Actively growing cultures with and without addition of methylene blue to the media showed similar growth curves, with no deviation in the viability and CFU counts due to presence of methylene blue in the media.

Mycobacterium aurum Cells Failed to Survive Under Nutrition Depletion Induced Stress Conditions

Mycobacterium aurum cells did not show constant CFU for extended periods of time when subjected to nutrition depletion conditions (Fig. 1b). The cells showed viability and maintained almost constant CFU for initial 10 days, followed by a 2 log decrease from day 10 to day 14. A sharp decline in CFU was observed from day 14 to day 17, leading to complete loss in cell viability, thereafter. Cell viability was further checked till 45 days post starvation (data shown only till 30 days) with no reversal of the cell viability. *M. aurum* cells appeared weakly acid fast after 1 week of nutrition depletion [Supplementary Fig. S1(c)], but maintained normal colony characteristics and retained orange pigmentation on plating till viability was observed.

Mycobacterium aurum Cells Did Not Survive Acid Induced Stress Condition

To check the effect of acidic stress on viability and growth characteristics of *M. aurum*, pre-adapted cells of *M. aurum* were subjected to acidic stress at pH of 5.5 and 4.5 (Fig. 1c). In control samples kept at pH of 6.5 (MB 7H9),

Fig. 1 **a** Growth and survival of *M. aurum* cells under hypoxia induced stress conditions. *M. aurum* cells were subjected to hypoxia induced stress conditions. 'f' indicates fading of blue color and 'd' indicates complete discoloration indicating hypoxic conditions. *M. aurum* cells were viable up to day 16, sharp decline in CFU was observed 16 day post sealing. Cells did not show constant CFU or survival for extended periods of time under hypoxic condition a characteristic of latent infection. **b** Growth and survival of *M. aurum* cells under nutrition depletion induced stress conditions. *M. aurum* cells were subjected to nutrition depletion induced stress. Cells showed viability and for initial 10 days, followed by a 2 log decrease from day 10 to day 14. A sharp decline in CFU was observed from

day 14 to day 17, followed by complete loss in cell viability thereafter. Cells did not maintain viability or survival for extended periods of time under nutrition depletion induced stress condition, a key feature of latent infection. **c** Growth and survival of *M. aurum* cells under acidic stress conditions. *M. aurum* cells were subjected to acid induced stress at pH 5.5 and 4.5. Bacilli showed progression towards death with sharp decline in CFU during acid stress. At pH 5.5, cells showed viability only for 2 days, with a sharp decline in CFU thereafter. After day 6 no viability was observed during the entire period of 30 days study. At pH 4.5, cells were initially viable but after 6 days of incubation under acidic stress conditions no viability was recorded (color figure online)

cells maintained a standard logarithmic phase thereafter cells entered a stationary phase followed by a phase of decline where viability count decreased slowly, showing a standard sigmoidal growth pattern. Bacilli exposed to acidic stress (pH 5.5 and 4.5) showed early progression towards death with sharp decline in CFU in comparison to control samples kept at pH 6.5. At pH 5.5, cells showed viability only for initial two days, with a sharp decline in CFU from day 3 to day 6. After day six no viability could be recovered during the entire period of 30 days study. Similar observation was recorded at pH 4.5, where no cell viability was observed after six days of incubation under acidic stress conditions. Alteration in staining characteristics (shorter appearance of bacilli and loss of acid fastness) was observed after five days under both acidic stress conditions [Supplementary Fig. S1(d)]. Interestingly, colony pigmentation of *M. aurum* was lost when subjected to acidic stress, with the colonies showing cream-yellow coloration instead of normal orange tone (Supplementary

Fig. S2). Such cells however, retained their acid-fast nature and were positive for ZN staining.

Discussion

TB is the second largest killer around the globe after HIV [18]. Remarkable incidence and prevalence rates of tuberculosis infection can be attributed to the competence of its etiological agent to persist as long-term asymptomatic form and simultaneously escape the host defense mechanisms. Normal anti-tubercular regimen is unable to tackle dormant forms of the bacilli, which poses a major hurdle in clearing the bacilli off the host body. Latency is a condition of equilibrium between the host and bacilli where, in response to the bacterial pathogenesis the host mounts a strong immune response leading to the formation of granulomatous structures surrounding a small population of dormant bacterial cells [19]. 'Granulomas' are

imperative for control of bacterial dissemination and are believed to be the seat of LTBI. Inside granulomas, bacilli are exposed to multiple stress conditions of oxygen deficit, nutrient starvation and acidic pH. Such physiological changes have been suggested to provide signals for entry into dormancy [20] or undergo genetic transformation for survival under such unfavorable conditions [21].

As processes and mechanisms pertaining to persistent infection may take long to manifest in humans thus, various in vitro and in vivo models of *M. tuberculosis* latent infection have been proposed. Furthermore, utilizing *M. tuberculosis* for in vitro model is associated with problem that it has a long generation time and requires BSL 3 facilities to handle. Hence, many rapidly growing mycobacteria (RGM) have been used as surrogate organism for in vitro and in vivo models of latent infection. A numbers of in vitro models of latency have been proposed exploiting the stress conditions prevalent inside granuloma as discussed above [1, 11], though none of them is able to fully recapitulate the *M. tuberculosis* latent infection. Thus, non-availability of a holistic model is a major obstacle in control and study of latent infection.

Mycobacterium smegmatis remains the most exploited surrogate organism to study LTBI, but outcomes from studies using this model organism have been unable to yield any conclusive solutions pertaining to latency or reactivation. The need of the hour is to explore the possibility of a better model organism for existing protocols that could be exploited for drug screening against LTBI, rapid screening of drug targets, and in deciphering various mechanisms pertaining to host-pathogen interactions explicitly during latent TB infection. Establishment of a novel and better model will help in the discovery of new drugs and drug targets against latent infection which will further help in shortening of current regimen time and complete elimination of the disease.

Among the known non-pathogenic fast-growing mycobacteria, *M. aurum* is reported to be the closer to *M. tuberculosis* in terms of mycolate components, presence of cyclopropane ring [10]. Pigmentation is considered to be an added advantage of using *M. aurum* so that contaminants could be easily marked off. The bacilli also possess a high level of resemblance to *M. tuberculosis* in terms of susceptibility profile against cell wall inhibitors. The ability of intracellular survival inside macrophages [22, 23] parallel to *M. tuberculosis* is another advantage to utilize *M. aurum* as a surrogate to study *M. tuberculosis* latency. *M. aurum* has been recently used as integrated surrogate model for screening of drugs against *M. tuberculosis* [12].

Thus, we aim to exploit a RGM, *M. aurum* as a surrogate organism to study *M. tuberculosis* latent infection. For this, *M. aurum* was subjected to three different granuloma specific stress conditions. Under hypoxia induced stress,

cells were initially viable but were unable to show constant CFU or enter state of non-replicating persistence. This behavior is contradictory to previous reports where *M. tuberculosis* [24], *M. smegmatis* [11] and *M. bovis* BCG [25] showed non-replicating persistence when subjected to gradual oxygen depletion induced hypoxia. Similarly *M. aurum* cells lost their viability when subjected to nutrition depletion induced stress, which is in contrast to behavior of slow growing *M. tuberculosis* [1] as well as rapidly growing mycobacteria *M. smegmatis* [26], where nutrient starvation-induced persistence is reported. Additionally, we subjected the bacilli to acid-induced stress condition reported to exist inside granulomatous lesions. Multiple studies have reported survival of *M. tuberculosis* under acidic stress, and correlated the altered gene expression during acidic stress to persistent state of *M. tuberculosis* [27, 28]. Acidic stress has been used as a one of the pivotal conditions in multiple stress model for *M. tuberculosis* [29]. *M. aurum* cells did not show constant CFU under acidic stress as well, and the cells were viable only for shorter duration of 4 days under the stress. During the study, cells lost pigmentation and pale colonies were observed, but retained their acid-fast nature on ZN staining.

Altered ZN staining is considered to be a key indicative of latent state of bacilli [30]. Alterations in the staining characteristics under latency associated stress conditions are common in different mycobacterial species [29, 31]. Under all three studied stress conditions viable bacilli showed altered ZN staining, with a fainter coloration or loss in coloration in comparison to deep purple-red color shown by actively replicating acid-fast bacilli. This might be due to stress conditions leading to changes in surface or cell wall characteristics. Under hypoxic and nutrition depletion stress conditions the behavior of *M. aurum* is in contrast to *M. tuberculosis* and *M. smegmatis* where cells enter a state of NRP and maintain a constant CFU for extended periods of time.

Thus, our study shows that *M. aurum* could not survive under any of the stress conditions related to *M. tuberculosis* latency. Hence, *M. aurum* cannot be used as surrogate to study *M. tuberculosis* latent infection. This leaves the doors wide open for exploration of other RGMs as suitable model organism. There is an urgent need of an organism which could serve as surrogate model of persistent infection. Such model may foster the exploration of new TB drugs targeted against latent form of *M. tuberculosis* which would aid in completely clearing the bacilli off the body and in turn shortening the unduly long current treatment regime. Apart from *M. smegmatis*, various other mycobacterial spp. like *M. phlei* [32] and *M. fortuitum* [33] (already used as surrogates in drug screening) can be researched as model organisms for *M. tuberculosis* latent infection, although detailed studies are required before an organism can be

considered as a holistic model mimicking all or most aspects of latent *M. tuberculosis* infection.

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Compliance with Ethical Standards

Conflict of interest Authors declare they have no financial/commercial conflicts of interest.

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