Protein synthesis is shutdown in dormant *Mycobacterium tuberculosis* and is reversed by oxygen or heat shock

Yan Min Hu, Philip D. Butcher, Kath Sole, D.A. Mitchison, A.R.M. Coates *

Department of Medical Microbiology, St. George’s Hospital Medical School, London SW17 ORE, United Kingdom

Received 23 September 1997; revised 5 November 1997; accepted 5 November 1997

Abstract

Oxygen-limiting conditions are critical to the survival of the bacteria in tuberculosis. *Mycobacterium tuberculosis* can survive anaerobiosis in vitro for long periods of time only after a gradual transition to a microaerophilic stationary phase. The underlying mechanism behind stationary phase adaption needs to be elucidated. The protein profiles of *Mycobacterium tuberculosis* during long-term stationary phase growth and under strict anaerobic incubation were monitored by 

\[
\text{[}^{35}\text{S]}\text{methionine labelling, SDS-PAGE and fluorography. These experiments have established that protein synthesis gradually decreased over 50 days in the long-term stationary phase cultures which were considered to be microaerophilic. There was an 80\% linear decrease in the level of total protein synthesis during the first 40 days of microaerophilic growth and then the rate of protein synthesis faded quickly. For the first time we have shown that total protein synthesis shutdown occurred when bacilli were incubated under further anaerobic conditions. Viability, estimated by cfu counts, remained constant during stationary phase growth and under anaerobic incubation. Furthermore, when oxygen was introduced into the anaerobic culture, protein synthesis restarted. Also heat shock at 45°C, 48°C and 50°C rapidly induced protein synthesis in stationary and anaerobic cultures. These data indicate that dormant bacteria shut down protein synthesis but remain responsive to specific stimuli which restore protein synthesis. In addition the dormant bacilli induced by anaerobiosis developed more heat resistance than non-dormant organisms.}

© 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: *Mycobacterium tuberculosis*; Dormancy; Anaerobiosis; Protein synthesis; Heat shock

1. Introduction

The World Health Organisation estimates that tuberculosis causes 3 million deaths annually and that one-third of the population is infected with *Mycobacterium tuberculosis* [1]. In the majority of those infected, the initial lesions heal, but *M. tuberculosis* often survives in a dormant form in the tissues for years and can eventually start to regrow and cause active disease. The existence of such dormant forms hinders the control of the disease. Furthermore, the need to treat patients for at least 6 months is probably due to the slow bactericidal activity of antituberculosis drugs against persisting dormant and semi-dormant bacilli in lesions [2]. Thus, an under-
standing of dormancy is particularly important in devising efficient control policies and improvements in chemotherapy.

Previous work indicates that oxygen tension plays an important role in the pathogenesis of tuberculosis including the possibility that low tension creates dormant bacilli. In rabbits and guinea pigs, reduction of atmospheric oxygen tension over a 3–4 week period inhibited the progress of experimental tuberculosis [3]. In humans, an inadequate oxygen supply to closed tuberculous lesions is usually thought to be responsible for the much smaller content of bacilli found in these lesions than in open cavities that communicate with air [4].

Wayne [5,6] investigated the oxygen requirements of tubercle bacilli which settle in a microaerophilic environment at the bottom of undisturbed liquid cultures. The bacilli in the deposits of logarithmic phase cultures were killed by transfer to strict anaerobic conditions. However, in the stationary phase, bacilli tolerated anaerobiosis. During the course of adaptation to anaerobiosis, metabolic shift-down occurred with a change from oxygen-dependent metabolic pathways to the glyoxylate cycle [7]. At the same time, metronidazole started to become bactericidal to the bacilli [8]. Recently Wayne and Hayes [9] have described a further model in which a down-shift of growth is needed to maximise anaerobic tolerance.

Only limited attempts have been made to characterise gene expression with in vitro models of dormant M. tuberculosis. The metabolic and adaptive changes occurring within dormant bacilli are essentially unknown. Recent work has demonstrated by 35S-methionine labelling and 2-D gel analysis that several proteins showed significant synthesis specifically in early stationary phase culture and that one predominant protein was identified as 16 kDa alpha-crystallin [10]. In order to establish the underlying mechanism behind stationary phase adaption and survival of M. tuberculosis we have quantitated the rate of protein synthesis and analysed protein profiles in an extended stationary phase model of dormancy [9] and in the subsequent transfer of these cultures to strictly anaerobic conditions. In addition, we have investigated the response of the dormant bacilli to heat shock and O2 depletion as markers of viability and metabolic integrity.

2. Materials and methods

2.1. Bacteria and culture

M. tuberculosis, strain H37Rv, was grown at 37°C in Middlebrook 7H9 medium containing 0.05% (v/v) of Tween 80 supplemented with 10% (v/v) ADC (Difco Laboratories). Samples of a 10 day mid-log-phase cultures were stored at −70°C. They were thawed and subcultivated once for 10 days before being inoculated 1:10 in fresh medium to form the experimental cultures. Stationary phase microaerophilic growth was achieved by incubating 10 ml cultures in 28 ml screw-capped bottles without shaking for up to 90 days. Cultures with loosened caps were incubated under strict anaerobic conditions, checked with anaerobic indicators (Oxoid), in a jar (GasPak 150 System, Becton Dickinson) in which H2 and CO2 were generated with GasPaks (Oxoid).

2.2. Cfu counts

From serial 10-fold dilutions of the experimental cultures, 100 ml samples were added to triplicate plates of Middlebrook 7H11 medium supplemented with OADC (Difco). Colony forming units (cfu) were counted after incubation of the plates in polythene bags for 3 weeks at 37°C.

2.3. [35S]methionine labelling of proteins and SDS-PAGE

Protein synthesis of M. tuberculosis at 37°C was examined by labelling with L-[35S]methionine (> 3.7×1013 Bq mmol−1, ICN Flow), added to a final concentration of 3.7×105 Bq ml−1. Unagitated cultures which had been incubated for 20–60 days were concentrated by carefully removing part of the supernatant so as to adjust viable counts to 8.2–8.3 log10 cfu ml−1, and 3 ml of each sample was then placed in a 28 ml sterile plastic universal container. One group of the cultures was then labelled by [35S]methionine in a conventional incubator, another group was placed in an anaerobic jar for various periods and were then labelled with [35S]methionine. A 24 h period of [35S]methionine labelling was performed for both incubated cultures. Labelling of protein synthesis during heat shock at
46°C, 48°C and 50°C was carried out in a water bath for 90 min followed by a further 30 min recovery at 37°C. Non-heat shock control cultures were labelled at 37°C for 2 h [11]. Radioactive labelling was terminated after a chase with 10 mM l-methionine and the bacteria were collected by centrifugation (12,000×g for 15 min at room temperature) and washed twice with phosphate-buffered saline (PBS) containing 1 mM l-methionine. Bacteria were lysed by vortexing at maximum speed for 5 min with an equal amount of glass beads (75 to 150 mm, Sigma) in distilled water. Proteins were solubilised and mixed with an equal volume of 2× sample buffer [12] and boiled for 5 min. The supernatants of the protein extracts were stored at −20°C before analysis on 12.5% (w/v) polyacrylamide gel electrophoresis (PAGE) in the presence of SDS [12]. After fixing, Coomassie blue staining, equilibration with Amplify (Amersham), and drying, gels were fluorographed for an appropriate time with preflashed X-ray film (Amersham, Hyperfilm MP) at 370°C.

2.4. Densitometric analysis

The fluorographs of SDS-PAGE were examined with a high-resolution laser Personal Densitometer SI (Molecular Dynamics) linked to ImageQuaNT software (Molecular Dynamics). Comparative quantitative data from [35S]methionine labelling imposed certain limitations because of unequal distribution of labelled methionine in different proteins, hence we calculated the relative rate of total protein synthesis comparing the total intensities in each lane.

3. Results

3.1. Protein synthesis under microaerophilic and anaerobic conditions

Metabolic labelling with [35S]methionine was used to monitor the protein synthesis of M. tuberculosis under microaerophilic and strict anaerobic conditions. Fig. 1 represents the radiolabelled protein profiles of long-term stationary phase cultures from 20 to 50 days. The amount of overall protein synthesis observed gradually decreased with prolonged stationary phase incubation. Densitometric analysis of the autoradiograph and others from three further independent experiments revealed a gradual decrease, evident at each 10 day interval, to 20% of initial density at 40 days then to 2% when bacilli reached late stationary phase at 50 days. Coomassie blue staining of the gel revealed that equal amounts of protein were loaded in each lane. The de novo protein profiles of the same stationary phase cultures incubated in an anaerobic jar for 24 h, 1 week and 3 weeks was also examined. In contrast to their microaerophilic controls the protein profile of M. tuberculosis synthesised after 24 h anaerobiosis was reduced by 96% of pre-anaerobic cultures, irrespective of duration of prior culture (one month exposure to X-ray film). Complete cessation of protein synthesis was observed at 1 week, again irrespective of duration of prior culture. Protein synthesis was undetectable during a further 3 weeks of anaerobic incubation (data not shown). The protein profiles of M. tuberculosis synthesised after one and three weeks

Fig. 1. SDS-PAGE analysis of proteins synthesised by M. tuberculosis H37Rv under microaerophilic condition. The autoradiograph shows [35S]methionine-labelled protein profiles of long-term stationary phase cultures from 20 to 50 days. Each lane represents proteins extracted from 8.2 log10 cfu bacilli. The autoradiograph represents three independent experiments.
anaerobiosis were not detectable even after a 2 month exposure to X-ray film. No decrease in viable counts was observed both during stationary phase and during anaerobic incubation of such cultures for a further 3 weeks.

3.2. Oxygen induced recovery of M. tuberculosis from dormancy

To investigate whether or not the dormant bacilli were capable of recovering from anaerobiosis when O$_2$ became available, the protein profiles of 35 day old stationary phase M. tuberculosis were examined by [$^{35}$S]methionine labelling with and without anaerobic incubation. [$^{35}$S]methionine was added to one tube during anaerobiosis for one week and to the other tube immediately after incubation, then placed in a conventional incubator for 24 h. Fig. 2 shows that protein synthesis could not be detected after 1 week of anaerobic incubation (lane 2), but when O$_2$ was reintroduced (lane 3), the rate of protein synthesis returned to 74% of the total proteins of the oxygenated control. The protein patterns obtained after exposure of anaerobic cultures to O$_2$ were similar to the control except that a few proteins were down-regulated (Fig. 2, arrows). This result was confirmed in three independent experiments.

3.3. Heat shock response of long-term stationary phase and anaerobically incubated M. tuberculosis

Another indicator of metabolic integrity and ability to respond to environmental stimuli was determined by heat shock of M. tuberculosis during long-term stationary phase and anaerobic incubation. Unagitated cultures of M. tuberculosis grown for 15 to 70 days were concentrated by removing of the supernatant. [$^{35}$S]methionine labelling was carried out during heat shock at 46°C and 48°C. Analysis of protein synthesis by SDS-PAGE and fluorography (Fig. 3) clearly demonstrated that although protein synthesis had gradually decreased during progression to stationary phase as shown in Fig. 1, the bacilli were still responsive to heat shock. In a 37°C to 46°C shift, strong induction of 90, 70, 65 and 19 kDa heat shock proteins was observed (Fig. 3A), while under more severe temperature stress (37°C to 48°C shift), additional protein bands with molecular masses of 55 and 14 kDa were induced (Fig. 3B) which was consistent with the previous observation that newly synthesised heat shock proteins were strongly up-regulated [13]. No difference in the heat shock protein synthesis profiles of M. tuberculosis was observed at both heat shock temperatures at different times in the growth of the cultures over 15 to 70 days.

The heat shock response of dormant bacilli, induced by anaerobiosis for 4 weeks, was carried out at 48°C and 50°C immediately after termination of anaerobic incubation. The dormant bacilli responded to heat shock. Major heat shock proteins were induced at 48°C and 50°C as shown in Fig. 3C. The heat shock proteins of the dormant bacilli were strongly up-regulated even at 50°C. Heat shock was performed with minimal disturbance to the cells and uptake of methionine was observed in contrast...
to non-heat shocked cells which showed no methionine uptake. However, the results were variable because the bacilli formed extremely solid clumps when the cells reached late stationary phase, which prevented uniform penetration of heat and $^{35}$S methionine into individual cells. To equally expose the cells to heat and $^{35}$S methionine, the cultures were briefly sonicated to make a single cell suspension. This procedure introduced oxygen into the cultures and induced a low amount of methionine uptake (see Fig. 3C). Heat shock induced a much higher level of methionine uptake. We examined the effect of 50°C over a period of 2 h on the viability dormant bacilli incubated anaerobically for 4 weeks and a 7 day mid-log-phase culture. Fig. 4 shows that after 2 h of exposure to 50°C, cfu counts of mid-log-phase culture decreased from 8.13 to 3.67, whereas the counts of anaerobically incubated culture dropped from 8.56 to 7.14 over the same period of time, indicating induction of thermotolerance.

4. Discussion

The patterns of proteins synthesised during the entry into the stationary phase induced by starvation have been analysed extensively in other bacteria. When *Escherichia coli* is subjected to carbon starvation the cells degrade their bulk proteins at an average rate of about 4–5% h$^{-1}$ [14], the rate of protein synthesis drops to about 20% of the initial rate during the first hour of starvation [15] and then remains roughly constant for at least the next 47 h. During carbon starvation for 6 days 50% of the cells lost viability. In contrast, we found that there was no change in viable counts during prolonged unagitated incubation and under strict anaerobic conditions.
Protein synthesis was, however, greatly reduced to about 2% of the original value, after 50 days unagitated incubation. Slowing down the rate of protein synthesis secures the viability of the bacilli, making the cells efficiently shut down the majority of their metabolic activities. Our results contradicted the recent finding that protein synthesis still occurred in stationary phase bacilli but at a low rate [10]. Transfer of cultures to strict anaerobic conditions resulted in a more rapid decrease and termination of protein synthesis. We propose that M. tuberculosis can persist under anaerobic conditions by entering a state of dormancy via the process of rapidly reducing or completely switching off protein synthesis in order to achieve a shutdown of cellular metabolic activity. The genetic regulation of the protein synthesis shutdown is unknown.

Our finding that the dormant bacilli responded rapidly to the introduction of oxygen by resuming normal protein synthesis patterns supports the view that oxygen triggers the emergence of bacilli from dormancy. In man, this could explain the replication of bacilli in lesions which are exposed to air [4]. Closed tuberculous lesions which have poor oxygen supply and low numbers of dormant bacilli communicate with air to form cavities in which larger numbers of bacilli are found.

We have demonstrated that the dormant bacilli induced by long-term stationary phase and anaerobiosis still retained their responsiveness to heat shock, which has been intensively investigated in M. tuberculosis [11,13]. We found that although protein synthesis in the stationary phase can be down-regulated and switched off under strict anaerobic conditions, the heat shock response persisted during the transition from the active to the dormant form. The same patterns of heat shock proteins were synthesised during heat shock in young, old and dormant bacilli. In Gram-negative bacteria, starved or stationary phase cells exhibit more resistance to heat shock, oxidative stress, and osmotic challenge than log-phase cells [16–18]. The dormant M. tuberculosis induced by anaerobiosis also developed a more heat resistant state. The heat shock proteins were up-regulated when the cells were subjected to heat shock at 50°C. This sharply contrasted with the log-phase M. tuberculosis in which heat shock protein synthesis ceased after 45 min during heat shock at 48°C [11]. A previous study showed that the stationary phase cultures survived at higher temperature than the log-phase cultures [19]. We found that the dormant bacilli were much more resistant to heat at 50°C than log-phase bacilli. These data suggest that despite quantitative metabolic shutdown, dormant bacilli retain their environmental responsiveness, indicating continuing metabolic integrity.

Acknowledgments

The authors are most grateful to Dr. L. Wayne for helpful discussions. The authors also thank the British Medical Research Council for financial support.

References


