Comparative proteomic profiles reveal characteristic *Mycobacterium tuberculosis* proteins induced by cholesterol during dormancy conditions

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Abstract

Cholesterol has been reported to play an important role during *Mycobacterium tuberculosis* infection and during its dormant state inside the host. We present the determination of proteomic profiles of *M. tuberculosis* H37Rv in the presence of cholesterol as the sole carbon source under exponential growth and in two in vitro dormancy phases (NRP1 and NRP2). Using 2D-PAGE, we detected that *M. tuberculosis* expressed a high diversity of proteins in both exponential and non-replicative phases. We also found that cholesterol was involved in the overexpression of some proteins related to sulfur metabolism (CysA2), electron transport (FixB), cell wall synthesis (Ald), iron storage (BfrB), protein synthesis (Tig and EF-Tu) and dormancy maintenance (HspX and TB 31.7). According to our results we propose that proteins Ald, BfrB, FadA5 and TB31.7 are likely to play a fundamental role during in vitro dormancy of *M. tuberculosis* in the presence of cholesterol, helping to counteract its intracellular hostile microenvironment.

INTRODUCTION

Tuberculosis (TB) remains a major health problem worldwide, with an estimated incidence of 10.4 million and 1.4 million deaths in 2015 [1]. TB is caused by *Mycobacterium tuberculosis*, a micro-organism that produces active TB in 5–10% of infected individuals. The remaining 90–95% of those infected individuals control but do not eliminate the pathogen [2]. In the bodies of infected individuals, *M. tuberculosis* faces adverse environmental conditions, to which it responds by entering a non-replicative state known as dormancy. In this dormant state, bacilli are able to survive and cause either a latent infection or produce active disease when the host’s immune system becomes compromised [3]. During latent infection, the bacilli are refractory to detection and treatment, and have low but detectable metabolic activity [4, 5]. Several *in vitro* models have been established to study dormant *M. tuberculosis*: one of the most studied is the hypoxia model of Wayne and Hayes [6], where the dormant state is induced by the gradual decrease of oxygen concentration. According to the oxygen saturation in the medium (1 and 0.06%, respectively), two non-replicating phases (NRP1 and NRP2) can be detected.

There have been several works in which *M. tuberculosis* dormancy has been investigated using transcriptomics or proteomics techniques. Among these, there are some reports on the proteomes of *M. tuberculosis* using the Wayne and Hayes model, where dextrose has been the carbon source employed [7, 8]. In these studies, the functional protein profiling revealed that 37.5% of proteins in the NRP1 phase were involved in degradation processes and 45.1% in NRP2 involved energy-producing metabolism. In...
general, \textit{M. tuberculosis} induced expression of about 1\% of its genes in response to dormancy [7].

Host lipids, in particular fatty acids and cholesterol, are used as energy sources by mycobacteria during intracellular active growth and dormancy. Vander Ven \textit{et al}. [9] demonstrated that cholesterol exerts a dominant effect on \textit{M. tuberculosis} metabolism when this pathogen is inside macrophages and they propound cholesterol metabolism as a target for efficient anti-TB drug development [9]. \textit{M. tuberculosis} uses lipids to modulate its interaction with the immune system through qualitative and quantitative changes in the lipid content of its cell wall. Other important lipid participation occurs during the survival of \textit{M. tuberculosis} inside phagosomes, where phagosome/lysosome fusion is inhibited by cholesterol and coronine 1 protein (TACO) participation [10, 11].

Recently, cholesterol utilization by mycobacteria as the sole carbon source has been studied by different groups [9, 10, 12]. One has demonstrated the crucial role of this sterol, through the expression of the \textit{Rv}1129c gene, during intracellular growth of \textit{M. tuberculosis} [12], and its function as a signal to trigger \textit{M. tuberculosis} expression of other genes, such as \textit{tgs}1, \textit{fad}D21, \textit{ksh}B and \textit{rpf}B, required for stress protection in the dormant state of the bacilli and for a favourable reactivation process [13]. Despite these studies, it is still unknown which proteins may be involved in aiding \textit{M. tuberculosis} in surviving and persisting when cholesterol is present.

Therefore, the aim of this work was to identify some of the proteins synthesized by \textit{M. tuberculosis} and to analyse the role they might play in the presence of cholesterol as the sole carbon source in order to mimic the lipid-hypoxic environment in which \textit{M. tuberculosis} needs to survive during its dormancy.

\textbf{METHODS}

\textbf{Bacterial strain and growth conditions}

\textit{M. tuberculosis} H37Rv was grown in Dubos medium (Difco Dubos Broth Base, USA) containing 10\% albumin-dextrose-catalase supplement (0.2\% dextrose, final concentration) (Becton Dickinson, MD, USA) and incubated at 37\°C until otherwise indicated. Growth in cholesterol was assessed by incubating at 37\°C in 7H10 agar (Becton Dickinson, MD, USA) previously described by our group [13]. The exponential phases of cultures were subjected to non-replicative persistence 1 (NRP1) and non-replicative persistence 2 (NRP2) as described by Garcia-Morales \textit{et al}. [9] demonstrating the crucial role of this sterol, \textit{M. tuberculosis} metabolism when this pathogen is inside macrophages and they propound cholesterol metabolism as a target for efficient anti-TB drug development [9]. \textit{M. tuberculosis} uses lipids to modulate its interaction with the immune system through qualitative and quantitative changes in the lipid content of its cell wall. Other important lipid participation occurs during the survival of \textit{M. tuberculosis} inside phagosomes, where phagosome/lysosome fusion is inhibited by cholesterol and coronine 1 protein (TACO) participation [10, 11].

Starting from the exponential phase (OD=0.4), the NRP1 phase (partial fading of methylene blue) was established after 5 days of incubation at 37\°C (in the presence of dextrose or cholesterol), while the NRP2 phase (complete fading of methylene blue) was reached after 15 and 20 days of incubation in the presence of dextrose or cholesterol, respectively, as described by Soto-Ramirez in 2017 [13]. Mycobacterial cells from each culture condition (exponential, NRP1 and NRP2) were used for c.f.u. ml\(^{-1}\) determination and protein isolation. All determinations were performed at least in quadruplicate.

\textbf{Preparation of mycobacterial cell extract and protein purification}

Mycobacterial cells were washed 10 times with 10 mM phosphate buffer, pH 7.4 and suspended in lysis buffer [10 mM phosphate buffer, pH 7.4 and a protease inhibitor mix (Complete Mini, Roche Diagnostics, USA)] at a concentration of 500 mg (wet cell mass) per 500 \(\mu\)l and then broken by mechanical disruption in the Fast Prep apparatus (BIO 101 THERMO) using sterile glass beads of 150–212 \(\mu\)m diameter (Sigma, USA) [14]. Each sample was subjected to eight cycles at high speed (6.5 m s\(^{-1}\)) for 15 s. The ruptured bacteria cells were centrifuged at 12 000 g for 5 min; pellets were discarded, and proteins were precipitated from the supernatant using the 2D Clean Up Kit (GE Healthcare Bio-Sciences, USA). The protein pellet was suspended in an appropriate volume of two-dimensional rehydration buffer [7 M Urea, 2 M Thiourea, 2\% CHAPS, 20 mM DTT and 1.2\% (v/v) immobilized pH gradient (IPG) buffer, pH 4–7 (GE Healthcare Bio-Sciences, USA)]. Protein concentration was estimated using the Bradford assay and protein concentration adjusted to 1.2 mg ml\(^{-1}\) (Quick Start Bradford 1X Dye Reagent, BIO-RAD).

\textbf{Two-dimensional gel electrophoresis (2D-PAGE)}

Isoelectric focusing (IEF) was carried out using the method of in gel rehydration [15]. IPG strips of pH 4–7 and 7 cm length (Immobiline DryStrips, GE Healthcare Bio-Sciences, USA) containing 150 \(\mu\)g of protein were rehydrated overnight at 20\°C. Strips were then focused on an Ettan IPG Phor III unit (GE Healthcare Bio-Sciences, USA) at 20\°C, using the following five-step programme: (I) 100 V for 1 h in constant mode; (II) 300 V for 1 h in constant mode; (III) 300–1000 V for 30 min in gradient mode; (IV) 1000–5000 V for 1.5 h in gradient mode and (V) 5000 V for 4 h in constant mode. The current limit was set at 50 \(\mu\)A per strip. After IEF, IPG strips were equilibrated for 10 min in equilibration buffer I [75 mM Tris-HCl, 6 M Urea, 29.3\% (v/v) Glycerol, 2\% SDS (w/v) and 65 mM DTT] followed by equilibrium buffer II (containing the same ingredients as buffer I but adding 135 mM Iodoacetamide instead of DTT) for 10 min.

Proteins were then separated on a 10\% SDS-PAGE (second dimension) in a vertical electrophoretic dual gel unit Mighty Small II (GE Healthcare Bio-Sciences, USA) at a constant voltage of 100 V for 2–3 h and gels were stained with Coomassie Brilliant Blue R250 to visualize proteins. Images
were acquired by G-Box scanner (Syngene, UK) using Gene Snap Software, V 6.08 (Syngene, UK). 2D gels were analysed using the Melanie 7.0 software (Gene Bio, Switzerland, www.genebio.com). Resultant composite images for dextrose and cholesterol cultures in all conditions tested were analysed for spot detection and spot matching, followed by differential expression analyses. Spot relative intensities were analysed statistically by RM two-way ANOVA followed by Tukey post-test using GraphPad Prism V 6.0 (USA, www.graphpad.com). The relative quantification was estimated as the ratio cholesterol/dextrose expression, always considering a value of ‘1’ as the expression obtained in the dextrose medium in the corresponding phase of growth. An equal amount of protein was loaded into each gel and experiments were repeated at least four times.

In-gel digestion and mass spectrometry (LC-MS/MS)

Protein spots of interest were excised from gels and were treated with freshly prepared 10 mM DTT in 50 mM NH₄HCO₃ at 56 °C for 45 min. After incubation, the DTT was replaced by the same volume of 55 mM Iodoacetamide. The dried gel pieces were incubated at 50 °C for 1 h with 50 mM NH₄HCO₃ containing 0.01 % of ProteaseMAX Surfactant (Promega, USA).

The resulting peptides were applied on an LC-MS system consisting of a fluid flow micro-chromatograph Accela with ‘splitter’ (1/20) and a mass spectrometer LTQ-Orbitrap Velos (Thermo-Fisher, San Jose, CA, USA) with an electrospray ionization system. Mass spectrometer calibration was performed with a solution of 10 calibrating molecules (Calmix, Thermo-Fisher, USA), which allows determinations with a better accuracy than 5 ppm. In the liquid chromatography system, a gradient of 10–100 % solvent B (acetonitrile 0.1 % acetic acid) at a flow rate of 400 nl min⁻¹ for 120 min was used on a 75×50 mm capillary column 2C18 (PicoFrit Proteopep New Objective, Woburn, MA, USA). Collision-induced dissociation (CID) and high energy collision dissociation (HCD) methods were used for peptide fragmentation, all spectra were acquired in positive mode detection, always considering a value of ‘1’ as the expression obtained in the dextrose medium in the corresponding phase of growth. An equal amount of protein was loaded into each gel and experiments were repeated at least four times.

RESULTS

Growth of M. tuberculosis in the presence of cholesterol

In order to mimic some nutrient conditions thought to be encountered by M. tuberculosis during in vivo infection and persistence [10, 16], we cultured M. tuberculosis H37Rv in Dubos medium supplemented with cholesterol. The OD₆₀₀ and c.f.u. ml⁻¹ data throughout the growth curve showed that cholesterol cultures always reached a higher cellular mass than those found in dextrose. By day nine, all cultures entered the stationary phase of growth (Fig. 1). In the two dormancy phases, the viability of mycobacteria was not significantly affected (Fig. S1, available in the online Supplementary Material) as previously reported [13].

Protein spot analyses of M. tuberculosis grown in cholesterol

In the three conditions studied, a differential protein expression was detected when cholesterol was used as a carbon source (Fig. 2). By 2D-PAGE-matching analysis, an average of 245 protein spots was detected in M. tuberculosis grown in cholesterol (see Fig. 3, discontinuous line), with a maximum of 274 spots found in NRP1 phase. Examination of four independent experiments using the Melanie 7.0 software revealed a total of 274 spots that were exclusively found (specific spots) when M. tuberculosis was grown in cholesterol (in all growth phases, grey bars), compared to a total of 163 spots that were exclusively found when M. tuberculosis was grown in dextrose medium (Figs S2 and S3). Most of the cholesterol spots were located mainly in a pI range of 4.6–6.0 and in a molecular weight range of 25–100 kDa. In the same condition, the number of specific spots was almost twofold more abundant in NRP1 than in NRP2 (Figs 2 and 3).

A total of 11 spots, which were differentially expressed among all growth conditions in cholesterol and dextrose media, are indicated by arrows in Fig 2 and S2, and were identified by LC-MS/MS. Details of the number of matched peptides and the percentage sequence coverage obtained for each protein spot are given in Table 1. The pI and MW of the identified protein were checked against the spot position on the gel. There were other spots that were differentially expressed among growth conditions, but some of them contained such a modification: oxidation (M). Mass tolerances for precursor ions and fragment ions were set to 20 ppm and 0.2 Da, respectively. In all cases match punctuations were less than 5 ppm. A discriminant score was performed for each analysed peptide; this value is the combination of two measurements of the search result. One is the expectation value for the peptide match (‘FDR’, a measure of the likelihood that a match is random) and the other is a ‘best peptide score’, which takes into account the fact that if a protein has been confidently identified in a sample, it is more likely that other peptides will be identified from the same protein (San Francisco, CA, USA; http://prospector.ucsf.edu/prospector).

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small amount of protein that analysis was not possible, and others were insufficiently defined on the gel.

Spot number 1 was identified as a probable trigger factor protein (Tig), spot number 2 corresponded to a thiosulfate sulfurtransferase (CysA2) and spot number 3 was identified as a probable iron-regulated elongation factor (EF-Tu). These three proteins were overexpressed during the exponential growth phase compared to the other conditions when cholesterol was used as the sole carbon source (Fig. 4).

Mass spectrometry analyses suggested that spots 4 and 5 (Table 1, Fig. 2) were chemically modified variants of the same L-alanine dehydrogenase (Ald). Accordingly, we joined together the two optical density values of both spots to obtain the corresponding bars shown in Fig. 4. This protein presented the highest expression value at the NRP1 phase of growth. Spot 6 was identified as the probable electron transfer flavoprotein (alpha-subunit) FixB, and was overexpressed in the NRP2 phase. Spot 7 corresponded to a bacterioferritin (BfrB), which exhibited the highest expression at the NRP2 phase. Similarly to Ald, spots 8 and 9 were identified as probable chemical variants of acetyl-CoA acetyltransferase (FadA5) (Table 1, Fig. 2). These proteins were overexpressed (in comparison to the exponential phase) during the in vitro dormancy and particularly presented the highest expression value during the NRP1 phase.

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**Fig. 1.** The *M. tuberculosis* growth rate is enhanced by cholesterol. *M. tuberculosis* was grown with either cholesterol (black lines) or dextrose (grey lines) as the sole carbon sources. Arrows indicate sampling points at exponential phase (Exp): 5 days for Exp-cholesterol, 6 days for Exp-dextrose. c.f.u. ml⁻¹ dextrose (grey circles); c.f.u. ml⁻¹ cholesterol (black circles); optical density OD₆₀₀ dextrose (grey square) and optical density cholesterol (black square). All experiments were carried out in quadruplicates.

**Fig. 2.** Differentially expressed proteins of *M. tuberculosis* grown in the presence of cholesterol. The panels show the protein profile of *M. tuberculosis* cultures at exponential phase (a) and two phases of in vitro dormancy: NRP1 (b) and NRP2 (c). Arrows correspond to the protein spot numbers shown in Table 1. Isoelectric point (pI); relative molecular mass (Mr).
Finally, spots 10 and 11 corresponded to the alpha crystallin HspX and to the universal stress protein TB31.7, respectively (Table 1, Fig. 2). Although the last three proteins (FadA5, HspX and TB31.7) were overexpressed in the dormancy model of Wayne and Hayes, TB31.7 and HspX were exclusively detected during the NRP1 and NRP2 phases.

**Comparative protein expression of M. tuberculosis grown under different carbon sources**

In relation to the protein spot analyses of *M. tuberculosis* grown in dextrose, proteins EF-Tu and Ald, were overexpressed at the exponential phase compared to other phases. On the other hand, FadA5 showed the highest expression in NRP1, and TB31.7 was only detected at NRP1 and NRP2 phases (Fig. S4).

In order to compare the relative abundance of the nine proteins differentially expressed in both carbon sources, (cholesterol and dextrose), we merged the image of each spot for both conditions using Melanie 7.0 software. The relative quantification was analysed and expressed as the ratio cholesterol/dextrose, always considering the value 1 as the expression obtained in the dextrose medium (Fig. 5). In relation to dextrose, Ald, BfrB and FadA5 proteins were overexpressed in cholesterol in all growth conditions (Fig. 5). In parallel, HspX was overexpressed in NRP1 and TB31.7 during the NRP2 phase of *in vitro* dormancy. While Tig was the only protein overexpressed in cholesterol at the exponential phase, compared to the other growth conditions, proteins CysA2, EF-Tu, FixB, BfrB, FadA5 and TB31.7 were overexpressed at the NRP2 phase (Fig. 5).

**DISCUSSION**

**The role of cholesterol on M. tuberculosis protein expression under different culture conditions**

It has been reported that cholesterol plays a fundamental role during the acquisition of the non-replicative *M. tuberculosis* phenotype using an *in silico* model of granuloma formation [17], as well as in pathogenesis and virulence [16, 18, 19]. In our *in vitro* study, *M. tuberculosis* was cultivated during its active growth (exponential phase) and during dormancy (NRP1 and NRP2 phases of the Wayne and Hayes model) to evaluate the expression of proteins in the presence of cholesterol. Overall, some changes in the bacterial proteome were identified. In particular, in the NRP1 phase, we detected more proteins (*n*=274) than in the exponential phase (*n*=243) (Fig. 3). These conditions together (cholesterol + NRP1) may stimulate *M. tuberculosis* to synthesize a larger amount of and a greater diversity of proteins to adapt to the stressful intracellular environment of its host, than it does in the exponential phase [20, 21].

Starck *et al.* [7] compared the proteome of *M. tuberculosis* Harlingen strain under aerobic and anaerobic conditions in the presence of palmitic acid and found that approximately 1% was expressed specifically in the absence of oxygen [7]. This is similar to our results found in NRP2 (anaerobic phase of the *in vitro* dormancy model of Wayne and Hayes), where we detected 59 *M. tuberculosis* specifically expressed proteins, namely, 1.4% of its proteome. This suggests that cholesterol has a similar effect to that observed with the fatty acids studied by Starck *et al.*, during adaptation of *M. tuberculosis* to dormancy.

**Fig. 3.** Proteome analysis of *M. tuberculosis* grown on cholesterol as the sole carbon source. Each bar represents the total and specific (exclusively found in each phase) number of protein spots predicted by the gel-matching analyses (see Methods for details) of the exponential (Exp) and of the *in vitro* dormancy phases (NRP1 and NRP2). A discontinuous line shows the average number of protein spots found in the presence of cholesterol.
Additionally, during NRP1 and NRP2 phases, we detected the presence of several low molecular weight proteins (10 kDa to 25 kDa) (Fig. 2). This data coincides with previous reports on the weight of a large number of chaperone proteins (Proteome 2D-PAGE Database, http://web.mpib-berlin.mpg.de/cgi-bin/pdbs/2d-page/exten/overview.cgi?gel=16) and some other proteins related to the methylcitrate cycle, where energy is obtained from propionyl-CoA degradation, one of the main components generated during the catabolism of cholesterol [12].

Although 2D electrophoresis and MS/MS presented some disadvantages in comparison with other proteomic approaches (e.g. such as LC-MS/MS), we were still able to identify specific proteins that demonstrated a differential expression in response to cholesterol at dormancy. Therefore, in the near future, these proteins might be investigated using further in-depth protein techniques, in a one-by-one protein fashion. Nine *M. tuberculosis* proteins that expressed differences during the two growth conditions studied were identified: Tig, CysA2, EF-Tu, Ald, FixB, BfrB, FadA5, HspX and TB31.7.

Tig is a chaperone molecule that participates in the initial protein-folding steps [22]. In our study, a decrease in the expression of this protein was observed in NRP1 and NRP2 (Fig. 4). By contrast, in our work we found a decrease of the expression found in the exponential phase) similarly to the decrease in EF-Tu expression was reported when using palmitate as the carbon source [28], M. tuberculosis metabolizes lipids, such as cholesterol or fatty acids, a decrease in CysA2 expression is established during NRP2 phases, similar to that reported by Rodríguez et al. [26] in the presence of fatty acids as the sole carbon source [26]. Therefore, we might conclude as a hypothesis that when *M. tuberculosis* metabolizes lipids, such as cholesterol or fatty acids, a decrease in CysA2 expression is established in order to avoid the resultant harmful reductive stress.

It has been reported that the *M. tuberculosis* elongation factor Tu (EF-Tu) promotes the aminoacyl-tRNA union to the A-site of the ribosomes during protein synthesis. This factor is activated by the serine-threonine-protein kinase PknB, that in turn promotes cellular division and the hypoxia stress response in the presence of dextrose [27]. In our work, in the presence of cholesterol, EF-Tu expression was decreased during in vitro dormancy (compared to the expression found in the exponential phase) similarly to the Tig expression pattern observed and reported above, supporting the important role of both in the protein synthesis process during bacterial active cell division.

When comparing phases NRP1 and NRP2, we detected an increase in EF-Tu protein expression during phase NRP2. Since an increase in EF-Tu expression in the presence of a high Fe²⁺ concentration and during hypoxia has been reported when using palmitate as the carbon source [7, 28], we suggest that cholesterol in NRP2 also contributes to the increase of intracellular iron accumulation. The functional advantages in comparison with other proteomic approaches [12].

**Table 1. M. tuberculosis** proteins upregulated in the presence of cholesterol and identified by LC-MS/MS

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Identified protein*</th>
<th>Identified protein’s ORF number</th>
<th>pI Exp/Theo</th>
<th>Mr, (kDa) Exp/Theo</th>
<th>No. of matched peptides†</th>
<th>% Sequence coverage‡</th>
<th>Best disc score‡</th>
<th>FDR§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Probable trigger factor protein (Tig)</td>
<td>Rv2462c</td>
<td>4.2/4.2</td>
<td>54/50.62</td>
<td>18</td>
<td>42.9</td>
<td>6.41</td>
<td>3.9e-9</td>
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<tr>
<td>2</td>
<td>Thioulate sulfurtransferase (CysA2)</td>
<td>Rv0815c</td>
<td>5.2/5.1</td>
<td>31/31.01</td>
<td>5</td>
<td>21.7</td>
<td>2.89</td>
<td>2.1e-4</td>
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<tr>
<td>3</td>
<td>Probable iron-regulated elongation factor (EF-Tu)</td>
<td>Rv0685</td>
<td>5.5/5.3</td>
<td>45/43.60</td>
<td>31</td>
<td>77</td>
<td>6.79</td>
<td>1.2e-9</td>
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<td>4</td>
<td>1-alanine dehydrogenase (Ald)</td>
<td>Rv2780</td>
<td>6.1/5.8</td>
<td>40/38.71</td>
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<td>35.8</td>
<td>6.82</td>
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<td>1-alanine dehydrogenase (Ald)</td>
<td>Rv2780</td>
<td>6.0/5.8</td>
<td>40/38.71</td>
<td>12</td>
<td>34.5</td>
<td>5.39</td>
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<td>Probable electron transfers flavoprotein (alpha-subunit) (FixB)</td>
<td>Rv3028c</td>
<td>4.5/4.7</td>
<td>30/31.70</td>
<td>11</td>
<td>73.9</td>
<td>6.69</td>
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<td>7</td>
<td>Bacterioferritin (BfrB)</td>
<td>Rv3841</td>
<td>4.8/4.7</td>
<td>18/20.44</td>
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<td>48.6</td>
<td>7.09</td>
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<td>8</td>
<td>Acetyl-CoA acetyltransferase (FadA5)</td>
<td>Rv3546</td>
<td>5.6/5.4</td>
<td>43/41.33</td>
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<td>30.7</td>
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<td>9</td>
<td>Acetyl-CoA acetyltransferase (FadA5)</td>
<td>Rv3546</td>
<td>5.8/5.4</td>
<td>44/41.33</td>
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<td>28.4</td>
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<td>10</td>
<td>Heat shock protein (HspX) (alpha-crystallin homolog) (14kDa antigen) (HSP16.3)</td>
<td>Rv2031c</td>
<td>5.0/5.0</td>
<td>16/16.23</td>
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<td>Universal stress protein family protein (TB31.7)</td>
<td>Rv2623</td>
<td>5.7/5.5</td>
<td>32/31.65</td>
<td>14</td>
<td>45.1</td>
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</table>

*The identification protein name and identified protein’s ORF number are listed according to the TubercuList database (http://tuberculist.epfl.ch/).

†Data details are shown in Table S1.

‡This was calculated from peptide value scores (values related to a protein that has been confidently identified in a sample).

§FDR, false discovery rate (see Methods).
relation between this sterol and Fe\(^{++}\) remains to be determined.

Bacterioferritin protein (BfrB) has been involved in \textit{M. tuberculosis} iron storage and has been reported to be necessary for \textit{M. tuberculosis} resistance strategies against host defences [29–32]. In addition, iron storage is crucial for \textit{M. tuberculosis} dormancy; loss or decrease in this function avoids bacillus persistence in murine models and increases its susceptibility to antibiotics [33, 34].

An increase in BfrB protein expression was registered during NRP2, which suggests that in this phase, iron is being stored to compensate for the absence of oxygen in the environment [35]. This increase was also observed by Gopinath et al. [25] who demonstrated an overexpression of mRNA levels during the non-replicative persistence phase in the presence of glycerol [25].

With respect to the proteins involved in \textit{M. tuberculosis} cholesterol catabolism, our study detected the enzyme FadA5, a thiolase, involved in \(\beta\)-oxidation of the aliphatic chain of cholesterol [36]. Other studies have observed that mutations in the gene coding for the FadA5 enzyme inhibits \textit{M. tuberculosis} from metabolizing the aliphatic chain of cholesterol and prevents the establishment of chronic infection in a pulmonary tuberculosis model [36, 37]. Moreover, Schaefer et al. [38] have demonstrated the potential of FadA5 as a therapeutic candidate against chronic \textit{M. tuberculosis} infection [38], opening up the possibilities of creating drugs that inhibit the synthesis of this enzyme. In our study, we observed that during \textit{in vitro} dormancy phases, this protein overexpression was higher than in the exponential phase, which supports the suggestion of some authors regarding the requirement of cholesterol for \textit{M. tuberculosis} in vitro dormancy entrance [10, 16, 39].

FixB has been described as an electron acceptor flavoprotein of many dehydrogenases at complex II of the cell respiratory chain [40] that maintain intracytoplasmic redox equilibrium. Compared to the exponential phase, in our work we found this protein was overexpressed in the NRP2 phase (Fig. 4). In relation to this, we can suggest that in these phases there should be an increase in the catabolism of the cholesterol aliphatic chain throughout the \(\beta\)-oxidation pathway, which in turn would produce an increase in reductive stress. Since this reductive stress is decreased, in order to maintain a redox balance inside the cell, the expression of FixB should increase.

Ald was another protein differentially expressed in this study. It synthetizes L-alanine from pyruvate, with the oxidation of NADH+H\(^{+}\), as was also reported in studies carried out with \textit{M. smegmatis} under standard hypoxia conditions (in the presence of dextrose as the carbon source) [41]. The enzyme was able to re-oxidize NADH+H\(^{+}\) when this
reduced molecule is found in excess, controlling the redox balance within the cell [25, 42]. We observed that the expression of Ald in the presence of cholesterol increased significantly in the NRP1 phase, contrary to what was observed when *M. tuberculosis* was cultured in dextrose (Figs 4 and 5). Thus, in our model (employing cholesterol and *M. tuberculosis*), Ald might be contributing to a drastic re-oxidation of NADH+H⁺ under stress conditions, such as the absence of oxygen, where its re-oxidation through the respiratory chain might be limited by oxygen concentrations. Another possible hypothesis for the overexpression of Ald would be the need to increase the thickness of the mycobacteria cell wall in order to tolerate the conditions of stress, and raising alanine synthesis would be a cellular alternative required in order to accomplish this.

Two additional proteins related to *M. tuberculosis in vitro* dormancy were identified: HspX and TB31.7. The first has been largely studied and has been described as a chaperone protein induced by hypoxia in *M. tuberculosis* and in *M. bovis* BCG using the Wayne and Hayes model [6], in the nutrient starvation model and in both guinea pig and mouse infection models [7, 23, 24, 35, 43, 44]. In our study, HspX was overexpressed in similar levels during NRP1 and NRP2 phases, confirming the importance of HspX as a molecular marker during in vitro dormancy, regardless of the source of carbon (dextrose or lipids).

TB31.7 protein, which has been associated with an in vivo dormancy regulatory function in *M. tuberculosis* (USP) family, Its overexpression has been reported during the same stress conditions as those when dosR/S/T are overexpressed (low oxygen levels, pH decrease and the presence of nitric oxide) [46]. Jain *et al.* [47] proposed this protein as a TB infection marker [47], based on the results they obtained in cerebrospinal fluid of patients with meningeal TB, where a significant overexpression of TB31.7 in TB patients (with latent and active TB) was detected compared to healthy individuals. In our study, the expression of this protein was only detected in both dormancy phases, mainly in NRP2, which suggests that the presence of cholesterol stimulates its expression particularly during the dormancy phases. This finding complements the proposal of Drumm *et al.* [45] regarding TB31.7 functioning as an intermediate of ATP-dependent signalling pathways in *M. tuberculosis* cholesterol catabolism [45]. In parallel, these investigators employed an *M. tuberculosis* mutant strain for the gene that codes for TB31.7 and observed a hyper-virulent phenotype that was unable to establish a chronic infection. By joining the Drumm *et al.* [45] results with ours, we strongly suggest that cholesterol might be working as a modulator of *M. tuberculosis* persistence within the host through the overexpression of TB31.7.

Expression of *M. tuberculosis* proteins in cultures with different carbon sources

When the overall expression of proteins identified with LC-MS/MS was compared between both carbon sources (dextrose and cholesterol), it was found that cholesterol stimulates the overexpression of the studied proteins in most cases (Fig. 5). These proteins were not only those
related with oxygen deprivation, (HspX and TB31.7), but also the ones related with cholesterol degradation (FadA5), iron storage (BfrB) and alanine synthesis (Ald). In particular, the overexpression of TB31.7 and BfrB (in our cholesterol model) has also been reported to be necessary for *M. tuberculosis* survival and installation of chronic infection using two animal models [33, 45].

**Comparative analysis of previously reported transcriptomic and proteomic results from *M. tuberculosis* grown under different stress conditions and those of our work**

In order to support our findings, we decided to compare our results (fold-changes in protein expression) with those obtained when *M. tuberculosis* was grown under different stress conditions. This comparison was analysed on two different expression levels: (1) examination of transcript levels (that correspond to some of our proteins) which were identified in some previous transcriptomic works [13, 26, 48], and (2) correlating our proteomic results with those obtained by Gopinath et al. [25] and Devasundaram et al. [49].

In relation to the expression of *M. tuberculosis* transcripts isolated during different stressing environments, such as the stationary phase of growth, hypoxia and patient sputum [13, 26, 48, respectively], genes *tig*, *cysA2*, and *tuF* were found to be down-regulated during the conditions already mentioned, compared to normoxia and these results correlated with ours at the protein level. In the same way, overexpression of genes *ald*, *hspX* and *TB31.7* matched that of their corresponding proteins found in our conditions (presence of cholesterol and hypoxia). Both of these comparisons allow us to validate and be confident in our protein expression results regarding these six proteins. The expression of the remaining three (FixB, BfrB and FadA5) found in our work do not coincide with the corresponding expression level of their transcripts (mentioned above). This discrepancy presented between expression of these three transcripts and our protein expression may rest on the fact that in bacteria, some variations in protein concentration are not solely related to transcriptional activity, but depend on protein stability and translation regulation [50] as has been demonstrated in *Lactococcus lactis*. Further work on...

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**Fig. 6.** Hypothetical proposed scenario for the in vitro protein expression of *M. tuberculosis* dormancy in the presence of cholesterol. See Discussion for details.
these processes related to the expression of proteins FixB, BrfB and FadA5 in mycobacteria would help to define if such is the case.

In order to corroborate the consistency of the representative protein expressions found in our work with those of other reports, we compared ours with results reported by Gopinath et al., which had determined a stress hypoxia/normoxia protein expression ratio in M. tuberculosis and had used a LC-MS/MS proteomic approach [25]. They found that proteins Ald, HspX, FixB and BrfB were overexpressed by M. tuberculosis in the same fashion that was found in our conditions. The only contrasting expression was found with CysA2, which we suggest should be further investigated. A similar agreement in the expression pattern of proteins Ald, HspX, FixB and BrfB was also found between our results and those reported by Devasundaram et al. [49], who worked with the same proteomic approach as we did (2D/MS) and who determined the sputum/normoxia protein expression ratio of M. tuberculosis [49]. We propose that these four proteins have an important role involved in M. tuberculosis survival when it faces stress conditions. Unfortunately, we could not compare the expression of our other four proteins (Tig, EF-Tu, FadA5 and TB31.7) with that of Gopinath’s or Devasundaram’s because either they were not reported or their expression was not detected.

Concluding remarks

In summary, a model for the in vitro protein expression of M. tuberculosis dormancy in the presence of cholesterol is suggested. In this hypothetical model (see Fig. 6), we propose the following scenario: once cholesterol is internalized, it is subsequently converted to pyruvate and propionyl-CoA by β-oxidation (FadA5), producing a reductive stress condition, which increases from NRP1 to NRP2. As there is a hypoxic environment, these reducing equivalents should be lowered to maintain bacterial homeostasis. In this sense, M. tuberculosis would use oxidative processes such as alanine synthesis (Ald) and Fe"++ uptake and storage (BrfB) in order to balance the reduced environment. As intracellular Fe"++ increases, the expression of some Fe-rich proteins like FixB and EF-Tu increased too. In addition, bacterial cells regulate the expression of other chaperones and stress proteins (Tig, HspX, TB31.7 and CysA2) to help maintain a stable redox intracellular balance in order to survive.

Finally, we suggest the implementation of further studies to gain more insight into the joint function of cholesterol metabolism and iron capture and storage, as key factors promoting M. tuberculosis persistence. These studies would contribute to further the understanding of the host–pathogen interaction of TB and would help propose new targets toward better control of this disease.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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