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Comparative Proteomic Analysis of Hypoxic Adaptation Reveals Conserved and Species-Specific Responses in *Mycobacterium tuberculosis* and *Mycobacterium bovis*

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Abstract

Tuberculosis, caused by members of the *Mycobacterium tuberculosis* complex, remains a major global health concern. While *M. tuberculosis* and *M. bovis* share similar pathogenic mechanisms, important differences in disease progression and adaptive responses have been observed. Hypoxia is a critical stressor during latent infection, however, the comparative molecular adaptations of these two species under oxygen-limited conditions remain incompletely understood. In this study, we used the Wayne

gradual oxygen depletion model to investigate the cellular proteomes of the reference strains *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv under hypoxic and aerobic conditions. Our analysis revealed that pathways involved in acyl-CoA generation constitute a conserved response to hypoxia in both species. Proteins associated with lipid metabolism and stress responses, particularly members of the DosRST regulon, emerged as hallmarks of hypoxic adaptation in both strains. Notably, a greater number of DosRST- and EHR-regulated proteins showed an increase in abundance in *M. bovis* than in *M. tuberculosis*. Lipid metabolism and cell envelope remodeling were central features of the hypoxic response in both species, consistent with observed changes in PDIM A and PDIM B levels. Both species also showed accumulation of proteins involved in molybdenum metabolism, whereas remodeling of nitrogen metabolism was a prominent feature specific to the *M. bovis* response.

These findings support the notion that pathogenic mycobacteria rely on lipid utilization to maintain energy homeostasis and redox balance under oxygen-limited conditions, and that cell envelope remodeling may contribute to long-term survival. The identified species-specific differences in metabolic adaptation and PDIM modulation provide new insights into the distinct physiological responses of *M. tuberculosis* and *M. bovis* during hypoxic stress.

Key words: *Mycobacterium bovis*, *Mycobacterium tuberculosis*, hypoxia, Wayne model, latency, proteomics

Introduction

Tuberculosis is primarily caused by members of the *Mycobacterium tuberculosis* complex (MTBC), among which *Mycobacterium tuberculosis* and *Mycobacterium bovis* are the principal etiological agents of human and bovine tuberculosis, respectively. Although humans and cattle are considered the primary hosts of *M. tuberculosis* and *M. bovis*, respectively, both species can cross host barriers and infect the alternate host [1, 2].

M. tuberculosis and *M. bovis* share similar routes of infection and pathogenic mechanisms, including aerosol transmission, pulmonary colonization, infection of alveolar macrophages, dissemination to lymph nodes and, occasionally, other organs, and the formation of granulomatous lesions in infected tissues. However, comparative studies in animal models, together with observations from naturally infected humans and cattle, have revealed important differences in disease progression, tissue damage, and pathological outcomes associated with these two species [3]

Comparative genomic analyses have further identified mutations and polymorphisms in key regulatory genes involved in stress adaptation and dormancy in pathogenic mycobacteria. These findings raise the possibility that *M. tuberculosis* and *M. bovis* may differ in their responses to host immune pressure and environmental stress, which may influence their capacity to establish and maintain latent or dormant infections.

During latency (dormancy), pathogenic mycobacteria encounter a range of environmental stresses, with hypoxia considered one of the most important [4]. Several in vitro hypoxia models have been therefore developed to investigate the adaptive responses of these pathogens during persistence [5–8]. In 1996, Wayne and Hayes developed a gradual oxygen depletion model that mimics the hypoxic conditions encountered by mycobacteria during prolonged persistence in the lungs [5]. Here, we used the Wayne model to characterize the proteomic adaptations of *M. bovis* and *M. tuberculosis* to hypoxia. Previous studies have established the proteomic changes associated with hypoxia in *M. tuberculosis* [9, 10]; however, considerably less is known about the corresponding response in *M. bovis*.

In a previous study, we used the Wayne model to identify the proteins that the *M. bovis* NCTC10772 strain preferentially accumulate under hypoxia [11]. Several proteins belonging to the DosRST regulon showed greatest increases in abundance under hypoxia, together with proteins involved in nitrogen and lipid metabolism, purine biosynthesis, carbon metabolism, and anaplerotic pathways. These findings indicated that, like *M. tuberculosis*, *M. bovis* undergoes extensive proteomic remodeling in response to oxygen limitation.

In the present study, we compared the cellular proteomes of *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv, the reference strains of the two species, under hypoxic and aerobic conditions. We further examined hypoxia-induced remodeling of phthiocerol

dimycocerosates (PDIMs), key virulence-associated lipids of the mycobacterial cell envelope.

Materials and Methods

1. Sources of the strains

M. tuberculosis H37Rv was obtained from BEI Resources (Manassas, VA, USA). *M. bovis* NCTC10772 was kindly provided by Dr J. MacFadden, University of Surrey, Guildford, UK, while *M. bovis* AF2122/97 was kindly provided by Dr Sharon Kendall, Royal Veterinary College, London, UK.

2. Growth of *Mycobacterium* strains

The reference strains *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 and *M. bovis* NCTC10772 were cultivated in Middlebrook 7H9 broth. The *M. bovis* medium was supplemented with 0.4% glucose, 0.2% glycerol, 0.4 % pyruvate, 0.5% bovine serum albumin (BSA), and 0.025% Tween 80, whereas the *M. tuberculosis* medium was supplemented with 0.4% glucose, 0.5% glycerol, 0.5% BSA, and 0.025% Tween 80. Cultures were incubated at 37 °C with constant agitation until reaching an optical density at 600_{nm} (OD₆₀₀) of 0.4-0.6.

For hypoxia experiments, actively growing cultures were diluted to OD₆₀₀ of 0.05 and distributed into 60-mL screw-cap glass tubes equipped with O-ring seals. Tubes were filled to approximately 80% of their total capacity and maintained under continuous stirring. Oxygen depletion was monitored using an independent culture supplemented with methylene blue (1.5 µg/mL), which served as an indicator of oxygen availability. At selected intervals, optical density was recorded from three independent cultures. Serial dilutions were plated onto Middlebrook 7H10 agar supplemented with the appropriate supplemented for each species, followed by incubation at 37 °C to determine colony-forming units (CFUs).

For proteomic studies, hypoxic cultures were prepared following the same protocol described above. Complete oxygen depletion was confirmed after five days by discoloration of the methylene blue control culture. Cultures were then maintained

under continuous agitation for an additional 300 h under oxygen-restricted conditions. Parallel aerobic cultures were grown in triplicate using the same medium.

At the end of the incubation period, OD₆₀₀ values were recorded for all cultures. Bacterial viability was assessed by plating serial dilutions onto supplemented 7H10 agar, followed by incubation at 37 °C to determine CFUs.

3. Protein extraction and purification

Bacterial cultures were centrifuged at 4,000 rpm for 30 min at room temperature. Cell pellets were washed once with PBS and suspended in a lysis buffer (12.5 mM Tris-HCl pH 7.2, 7 M urea, 2 M thiourea, 1.5% sodium deoxycholate, 1.5% DTT, and protease inhibitor cocktail). Cell disruption was performed using a Precellys homogenizer (Bertin Technologies).

Following mechanical lysis, samples were centrifuged at 10,000 × g for 30 min at 4 °C. Proteins present in the supernatant were precipitated by adding five volumes of cold acetone, followed by overnight incubation at 4 °C. Precipitated proteins were collected by centrifugation at 10,000 × g for 30 min at 4 °C, resuspended in ultrapure water, quantified, and freeze-dried until use.

Each protein preparation corresponded to material obtained from 50 mL of bacterial culture. Protein integrity was assessed by PAGE analysis.

4. Protein identification by mass spectrometry

Following resuspension in 8 M urea, lyophilized samples were prepared for analysis according to the filter-aided sample preparation protocol [13]. Protein samples were transferred to 10 kDa molecular weight cutoff spin filters (Qiagen), where cysteine residues were sequentially reduced and alkylated with 10 mM DTT and 55 mM, respectively. The samples were subjected to overnight digestion with trypsin (Sequencing Grade, Promega) at 37 °C, using an enzyme-to-protein ratio of 1:25 (w/w). After digestion, peptides were collected by centrifugation and desalted using self-packed microcolumns in pipet tips [14]. For LC-MS/MS analysis, peptide mixtures were separated by nano-HPLC on an UltiMate 3000 system (Thermo Scientific) using an EASY-Spray™ reverse-phase column (50 cm length, 75 µm internal diameter; Thermo Scientific). Chromatographic separation employed solvent A, consisting of 0.1% formic

acid in water, and solvent B, consisting of 0.1% formic acid in acetonitrile. A linear increase in solvent B from 4% to 35% was applied over 150 min, with flow rate of 200 nL/min. Mass analysis was performed using an Exploris 240 Orbitrap mass spectrometer (Thermo Scientific) operated in data-dependent acquisition mode. MS spectra were acquired over a mass range of 200–2000 m/z, followed by higher-energy collisional dissociation (HCD) of the 20 most intense precursor ions. Protein identification and quantitation by spectral counting were performed using PatternLab for Proteomics V [15]. For database searching, target-decoy databases were generated by combining the proteome of *M. tuberculosis* or *M. bovis*, strains under investigation, obtained from UniProt, with corresponding decoy databases generated by reversing all protein sequences. This strategy enabled the estimation of the false discovery rate (FDR) during peptide-spectrum matching. The databases were also supplemented with the most common contaminants encountered in proteomics experiments. Methionine oxidation was considered a variable modification, whereas cysteine carbamidomethylation was specified as a fixed modification. A precursor mass tolerance of 35 ppm was used, and semi-tryptic peptide cleavage was permitted. The resulting protein identifications were filtered to achieve a false discovery rate of less than 1% at the protein level. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD083669.

5. Bioinformatics analysis

The complete set of proteins detected in *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 under hypoxic and aerobic conditions is provided in Table S1. Differential protein accumulation between hypoxic and aerobic conditions was assessed for each strain. Mass spectrometry data were normalized using Spectrum Count Unique (SCU) values. Briefly, a constant of 0.1 was added to each SCU value to account for zero counts. The resulting values were then normalized to the total SCU for each biological replicate and condition. Fold changes (FC) between hypoxic and aerobic conditions were subsequently calculated and expressed as log₂-transformed fold changes (log₂FC). Proteins were considered differentially accumulated when they exhibited a log₂FC ≥ 0.2 or ≤ -0.2, together with a nominal p-value < 0.05 and a FDR < 0.25. Statistical significance was assessed using an independent two-sample t-test based on three biological

replicates per condition. Multiple-testing correction was performed using the Benjamini–Hochberg procedure.

For downstream functional analysis, differentially accumulated proteins were classified into two groups according to their preferential accumulation under hypoxic or aerobic conditions. Functional enrichment analyses were performed using STRING (<https://string-db.org/>). Protein functional classification and categorization were based on the *M. tuberculosis* H37Rv genome annotation available in MycoBrowser (<https://mycobrowser.epfl.ch/>).

For comparative analyses, proteins showing the highest differential accumulation were selected using a more stringent threshold of $FC \geq 2$ (equivalent to $\log_2FC \geq 1$) and an adjusted p-value ≤ 0.25 . These criteria were used to identify proteins showing the highest accumulation under hypoxic conditions in *M. tuberculosis* H37Rv (Table 1) and *M. bovis* AF2122/97 (Table 2). Conserved proteins showing differential accumulation under hypoxic or aerobic conditions among *M. bovis* NCTC10772, *M. bovis* AF2122/97, and *M. tuberculosis* H37Rv were identified through comparative analysis of the corresponding protein datasets (Table 3).

6. Lipid purification and analysis

Cultures of *Mycobacterium* sp. were grown under hypoxic conditions using the Wayne model, as previously described. Bacterial pellets were resuspended in water, heat-inactivated at 80 °C for 1 h, and subsequently freeze-dried. Dry biomass was determined prior to lipid extraction. Total cellular lipids were isolated according to the method described by Stadthagen and collaborators [16], using a chloroform:methanol extraction. Lipid fractions were separated by thin-layer chromatography (TLC) on silica gel 60F254 plates, with approximately 0.26–0.28 mg of lipid material loaded per lane. Plates were developed with petroleum ether:ethyl acetate (98:2). Lipid bands were visualized by treatment with a $CuSO_4$ /phosphoric acid reagent, followed by heating.

7. Statistical analysis

Graphs were generated using GraphPad Prism (GraphPad Software, Boston, MA, USA). Data were analyzed by two-way analysis of variance (ANOVA), followed by Tukey's multiple-comparisons test.

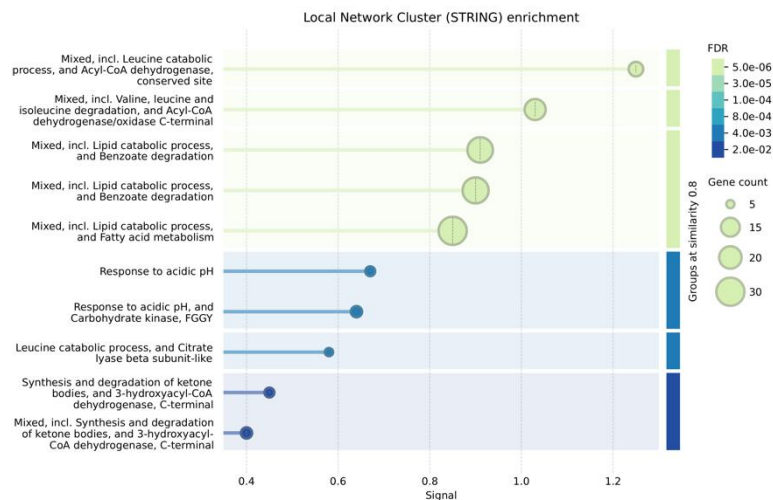
Results and Discussion

1. Proteins differentially expressed in *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 under hypoxic and aerobic conditions

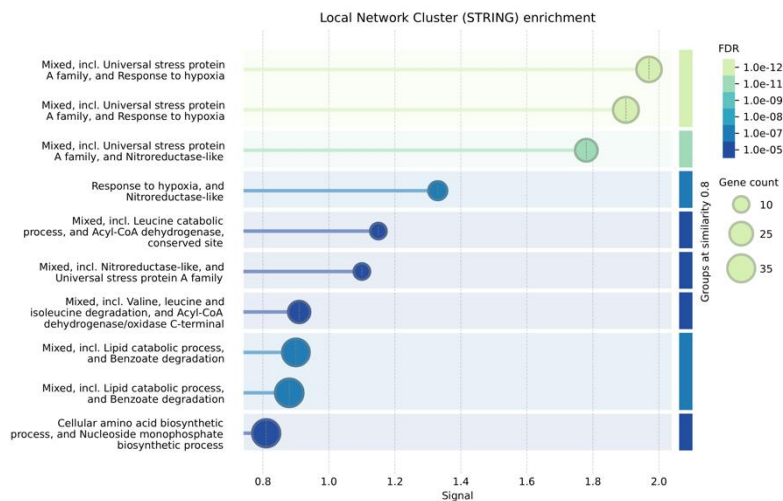
To perform a comparative proteomic analysis of proteins showing differential increases in abundance in *M. tuberculosis* and *M. bovis* under hypoxic and aerobic conditions, we first evaluated changes in protein abundance between hypoxia and aerobic growth in both species ($p_{adj} \leq 0.25$). Differential expression analysis identified 380 proteins in *M. bovis* AF2122/97 that were significantly more abundant under hypoxia, whereas 619 proteins showed significantly higher abundance under aerobic conditions. In *M. tuberculosis* H37Rv, 221 proteins showed higher abundance under hypoxia, while 358 proteins were more abundant during aerobic growth (Table S2).

STRING network analysis of proteins accumulated in hypoxic *M. tuberculosis* H37Rv cultures identified "leucine catabolic process," "acyl-CoA dehydrogenase activity," "lipid catabolic process," and "benzoate degradation" as the functional clusters with the strongest enrichment signal (Fig. 1A, Table S2).

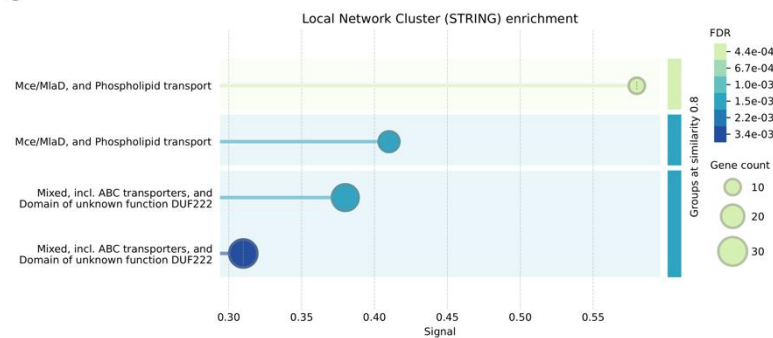
A



B



C



D

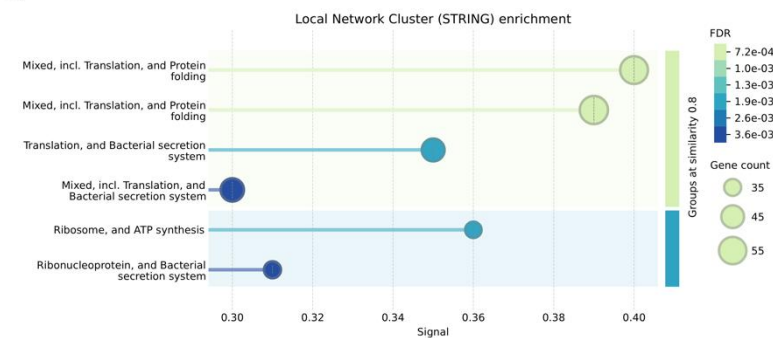


Figure 1. Functional enrichment analysis of differentially accumulated proteins under aerobic and hypoxic conditions in *M. tuberculosis* H37Rv and *M. bovis* AF2122/97. STRING local network cluster analysis was performed to identify functionally related proteins within each dataset. (A) Proteins more abundant under hypoxia in *M. tuberculosis* H37Rv. (B) Proteins more abundant under hypoxia in *M. bovis* AF2122/97. (C) Proteins more abundant under aeration in *M. tuberculosis* H37Rv. (D) Proteins more abundant under aeration in *M. bovis* AF2122/97. The x-axis represents the enrichment signal for each functional cluster, and circle size indicates the number of proteins associated with each cluster. Circle color represents the false discovery rate (FDR) of the enrichment analysis. Functional clusters are shown according to the annotations provided by the STRING database.

Among the proteins showing the greatest increases in abundance under hypoxia ($FC \geq |2|$, $padj \leq 0.25$) (Table 1), several were encoded within four candidate operons. The first corresponded to the *mymA* operon (*Rv3083–Rv3089*), whose expression is induced under acidic conditions and regulated through phosphorylation of the transcriptional regulator VirS by the Ser/Thr kinase PknK [17]. This operon encodes enzymes proposed to participate in fatty acid modification required for cell envelope maintenance [18]. The second operon comprised *Rv3550–Rv3553*, including the cholesterol degradation-associated hydratase *echA20*, consistent with the reported role of this genomic region in steroid ring and side-chain catabolism [19], as well as additional proteins putatively involved in lipid metabolism. The third corresponded to the *citE–scoA* operon (*Rv2498c–Rv2504c*), together with its associated transcriptional regulator *bkaR* (*Rv2506*). In addition, two members of the BkaR regulon, BkdA and BkdB, encoded within the upstream *pdhABC* operon, also showed increased abundance under hypoxia. These genes have been associated with branched-chain keto acid metabolism in mycobacteria [20]. Finally, the *echA7–fadE13* operon encodes enzymes involved in lipid metabolism, including the β -oxidation enzymes EchA7, FadE12, and FadE13, together with the acyl-CoA carboxylase subunits AccA2 and AccD2, suggesting coordinated regulation of fatty acid degradation and lipid biosynthesis.

In addition, seven transcriptional regulators (RegX3, Rv0196, PknK, Rv2324, Rv0485, Rv2506, and EthR), six proteins associated with the hypoxic response (AhpC, Hrp1, Rv1996, Rv2628, NarK2, and Rv0628c), and two putative S-adenosylmethionine-dependent methyltransferases (Rv0145 and Rv0830) were also among the proteins with

the highest accumulation under hypoxia. The accumulation of several of these proteins is consistent with previous reports of the *M. tuberculosis* response to hypoxic conditions [21–25].

Table 1. Proteins of *M. tuberculosis* H37RV showing the highest accumulation under hypoxia (FC $\geq$ |2|, adjusted p $\leq$ 0.25)

Functional Category	Proteins
Stress Response and Persistence	AhpC, Hrp1 (Rv2626c), Rv1996, Rv2628, Nark2, Rv0628c
Lipid Metabolism and acyl-CoA Metabolism	EchA7, EchA20, FadE12, FadE13, FadE19, AccA1, AccAa2, AccD1, AccD2, Rv3085, AdhD, LipR, Rv3553, Rv3552, Rv3087, MbtI, BkdA, CitE, ScoA, ScoB, Lat, Rv2499c, Rv2506
Signal Transduction and Regulation	Regx3, PknK, EthR, Rv0196, Rv2324, Rv0485, Rv1364c, Rv2606
Energy Metabolism and Intermediary Metabolism	PncA, Rv0245
Proteins with Conserved Domains	RpsK, EphA, Rv0830 Rv1779c, Rv0145
Proteins of Unknown	Rv2327, Rv0572a, Rv0441c, Rv0274, Rv1148c, Rv2125, Vapc47

STRING analysis of proteins from hypoxic *M. bovis* AF2122/97 cultures identified two distinct clusters showing the strongest enrichment signals in this strain: "Universal stress protein A family, and Response to hypoxia" and "Leucine catabolic process, and acyl-CoA dehydrogenase, conserved site" (Fig. 1B).

Among the proteins showing the highest increases in abundance under hypoxia (FC $\geq$ |2|; n = 115)(Table 2), several were encoded within seven candidate or confirmed operons. Three of these, *pdhABC* (*bkdA-C*), *citE–Rv2504c* and *echA7–fadE13*, also showed markedly increased abundance in H37Rv. In addition, we identified proteins encoded in a genomic region linked to NAD⁺ salvage under hypoxia (*Rv0571c–Rv0573c*), proteins involved in proline degradation (Rv1187, Rv1188, and Rv1190), and proteins from three additional genomic regions or candidate operons encoding stress-associated proteins. The proteins showing the greatest increases in abundance also included

canonical members of the DosRST dormancy regulon, namely Rv0080, HspX (Rv2031c), Rv2030c, Rv2032, Rv2028c, Rv2003c, Rv2004c, Rv2005c, Rv2624c, Rv2625c, Rv2626c, Rv2627c, Rv2628, Rv2629, Rv2631, Tgs1 (Rv3130c), Rv3131, Rv3134c, and Rv1738, as well as the sensor kinase DevS (Rv3132c). Other identified proteins belong to the enduring hypoxic response (EHR), the ferredoxin FdxA, Hely, FdhF, CydC, and the *Rv0571c–Rv0573c* gene products, further supporting adaptation to prolonged oxygen limitation (Table 2).

Table 2. Proteins of *M. bovis* AF2122/97 showing the highest accumulation under hypoxia ($FC \geq |2|$, adjusted $p \leq 0.25$)

Functional Category	Proteins
Hypoxia Response and Stress Adaptation	Rv0080, HspX, FdxA, Rv1738, Rv1996, Rv2003c, Rv2004c, Rv2005c, Rv2028c, Rv2030c, Rv2032, Rv2624c, Rv2625c, Rv2626c, Rv2627c, Rv2628, Rv2629, Rv2631, Rv3130c, Rv3131, Rv3134c, Rv0079, CtpF (Rv1997)
Lipid metabolism, acyl-CoA metabolism and Cell Envelope Biosynthesis	AccA1, AccA2, AccD1, AccD2, Plsc, FadD8, FadE13, FadE22, FadE32, EchA20, ScoA, ScoB, Mesj, LysX, Rv2499c, Rv2500c, Rv2505c, KshA (Rv3526), NagA (Rv3332), Rv3087, Rv0972c
Energy Metabolism and Intermediary Metabolism	Fdhf, Ribf, Thid, Malq, Cdd, Rv0573c, RocA, Rv1188, GlpD1, Rv3559c, Rv3784, Rv1774, AdhA (Rv1862), Rv1928c, ArgA (Rv2747), NuoJ (Rv3154), Cyp136, AtpB (Rv1304)
Transport and Membrane-Associated Proteins	Oppb, Dppa, Cyst, LpqC, CydC, LpqA, RfbD, Rv1057, Rv1456c, Rv1707
DNA Replication, Repair, Transcription and Translation	HelY, AlkB, Gid
Regulatory Proteins	Pknk, Rv0792c, EthR (Rv3855)
Conserved Hypothetical Proteins / Proteins of Unknown Function	Rv0274, Rv0375c, Rv0485, Rv0885, Rv0897c, Rv1219c, Rv1289, Rv2332, Rv2522c, Rv2575, Rv2826c, Rv2964, Rv3544c, Rv3565, Rv3895c, Mb0587CA, Mb0809c, Mb1920c, Rv0311, Rv0552, Rv0976c, Rv1069c, Rv1835c, Rv3127, Rv1084, Rv1190, Rv1290c, Rv1342c, Rv0571c, Rv0572c, Rv2240c, Rv3226c, Rv0857, Rv1225c, Rv2119, VapC32

Overall, both strains shared enrichment in lipid metabolism, branched-chain amino acids (BCAA) catabolism, and pathways associated with adaptation to low-oxygen conditions, highlighting common mechanisms of persistence under hypoxic stress. However, AF2122/97 showed stronger enrichment of proteins involved in the DosRST-mediated hypoxic response, persistence, stress adaptation, and cell envelope remodeling, whereas H37Rv showed more pronounced enrichment of catabolic pathways, particularly those involved in lipid degradation.

Under aeration, STRING analysis of the H37Rv proteome revealed significant enrichment of Mce/MlaD proteins, phospholipid transport, and ABC transporter-associated pathways (Fig. 1C). Among the proteins showing the greatest increases in abundance under aeration compared with hypoxia ($FC \geq |2|$; $padj \leq 0.25$, Table S2), proteins involved in host–pathogen interactions predominated. These included numerous PE/PPE family proteins (PE12, PE27A, PE31, PE_PGRS38, PPE3, PPE4, PPE19, PPE20, PPE31, PPE32, PPE33, PPE40, PPE51, PPE60, and PPE62), secreted and immunodominant antigens (Apa, EspA, EspJ, EsxD, CFP2, Mpt63, and Mpt70), and Mce4 proteins implicated in cholesterol uptake and utilization (Rv3492c, Mce4A, Mce4C, and Mce4F). Proteins associated with toxin–antitoxin systems (VapB15, VapB26, VapB31, VapB33, VapB37, VapB40, VapB44, VapB9, VapC27, and VapC35), transcriptional regulation (Rv0516c, Rv1816, Rv0653c, Rv3249c, and Rv3160c), and lipid metabolism (EchA2, FadD5, FadD21, FadH, Cyp125, LipO, and LipD) were also highly represented. Collectively, these findings indicate activation of a virulence-associated physiological program characterized by enhanced host interaction, intracellular adaptation, persistence, and utilization of host-derived lipid substrates.

In AF2122/97, STRING analysis revealed enrichment of proteins involved in translation, ribosome biogenesis, protein folding, and ATP synthesis, which represented the most significant functional clusters (Fig. 1D). Among the proteins showing the greatest increases in abundance under aeration ($FC \geq |2|$; $padj \leq 0.25$; Table S2), the most prominent functional groups included secreted proteins (Apa, EspA, EspC, EspJ, EspG3, EsxL, EsxM, EsxN, CFP2, CFP21, Mpt53, Mpt63, and Mpt64), Mce proteins—particularly members of the Mce1 complex (Mce1A, Mce1B, Mce1C, Mce1D, Mce1F)—Mce4C, and the transcriptional regulator Mce2R. Other prominent groups comprised regulatory proteins and signal transduction components, including SigF, SigD, RseA, RskA, TrcR, NarL, PknL, PknDb, PtkA, ArgR, VirS, NmtR, and HspR; extensive toxin–antitoxin systems, including multiple VapBC, MazEF, RelJ, ParD, and YdcDE family members; and proteins involved in lipid metabolism and lipid biosynthesis, including Pks11, FadD5, FadD11, FadE20, LipC, LipD, LipL, Cdh, Cyp125, and Cyp138.

Overall, despite species-specific differences in their global STRING enrichment profiles, H37Rv and AF2122/97 showed highly similar functional patterns among the proteins with the greatest increases in abundance under aeration. Both strains exhibited

increased abundance of proteins associated with secretion, Mce-mediated host interaction, toxin–antitoxin systems, transcriptional regulation, and lipid metabolism. These shared patterns support a conserved physiological response to aerated growth that are consistent with adaptation to host-associated environments.

2. Shared proteins in aerobic and hypoxic between H37Rv and AF2122/97

Among the proteins significantly enriched under hypoxia in both *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv, 85 were shared between the two species (Table S3). STRING network analysis of these shared proteins revealed three major functional clusters associated with the hypoxic response (Fig. 2). The first cluster comprised proteins involved in BCAA degradation and proteins containing an acyl-CoA dehydrogenase/oxidase C-terminal domain. The second cluster includes enzymes of lipid metabolism. The third cluster consisted of proteins associated with dormancy and adaptation to latency, including members of the DosRST regulon and other stress-response proteins implicated in long-term survival under oxygen-limiting conditions.

One of the most striking findings was the coordinated increases in abundance of proteins involved in acyl-CoA metabolism under hypoxia. In both *M. tuberculosis* and *M. bovis*, proteins encoded by the *bkdA-C* and *citE-scoB* operons showed increased abundance under hypoxia. These operons encode enzymes involved in BCAA catabolism and have been proposed to constitute a common regulon controlled by the transcriptional regulator BkaR (Rv2506) [20, 26]. In addition, proteins encoded by the *echA7-fadE13* operon, which comprises enzymes involved in acyl-CoA metabolism, also showed increased abundance under hypoxia in both species [27]. Furthermore, Rv2499c, a multifunctional lyase/thioesterase that participates in itaconate dissimilation and generates acetyl-CoA and pyruvate, was also more abundant under hypoxia [28].

The coordinated increase in abundance of these proteins suggests that hypoxia induces a metabolic reprogramming that enhances the capacity to generate central acyl-CoA intermediates, including acetyl-CoA, propionyl-CoA, and related acyl-CoA derivatives. These metabolites act as key metabolic hubs that integrate carbon and energy metabolism while serving as essential precursors for the biosynthesis of complex cell-envelope lipids, including methyl-branched lipids such as PDIMs. Thus, the increased abundance of enzymes involved in acyl-CoA production may contribute to maintaining

metabolic homeostasis and providing carbon precursors for lipid biosynthesis and remodeling during adaptation to oxygen limitation.

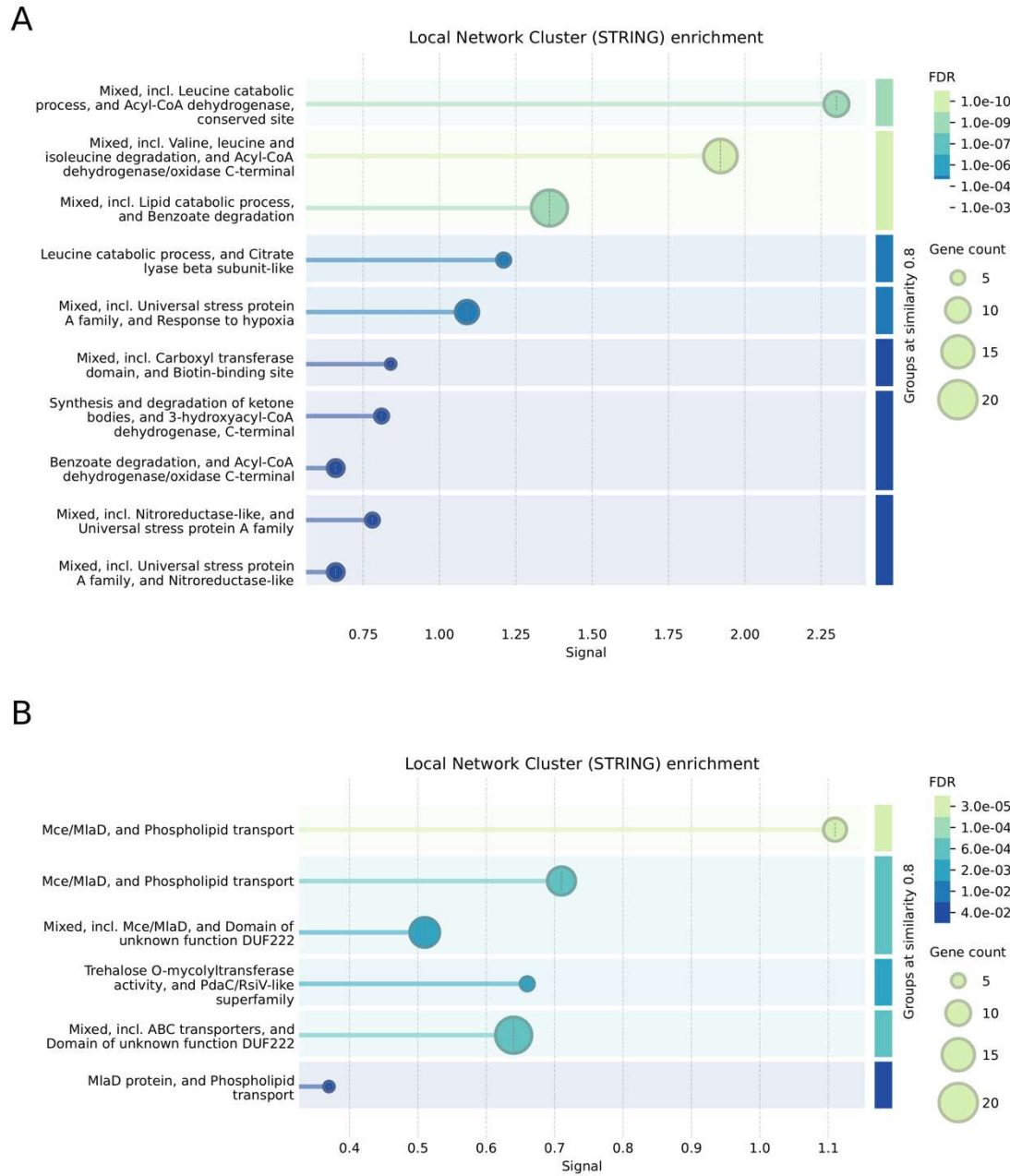


Figure 2. Functional enrichment analysis of shared proteins accumulated under aerobic and hypoxic conditions between *M. tuberculosis* H37Rv and *M. bovis* AF2122/97. STRING local network cluster were performed to identify functionally related proteins within each dataset. (A) Proteins shared between the two species that accumulated under hypoxia. (B) Proteins shared between the two species that accumulated under aeration. The x-axis represents the enrichment signal for each functional cluster, and the circle size indicates the number of proteins associated with each cluster. Circle color represents the false discovery rate (FDR) of the

enrichment analysis. Functional clusters are defined according to the annotations provided by the STRING database.

Under aerated conditions, both H37Rv and AF2122/97 showed increased accumulation of proteins (N=158), which clustered into three major functional groups: Mce/MlaD and phospholipid transport; trehalose O-mycolytransferase activity and PdaC/RsiV-like superfamily; ABC transporters and the domain of unknown function DUF222 (Fig. 2; Table S3). This conserved functional profile indicates that both strains respond to oxygen availability through comparable physiological adaptations, potentially enhancing metabolic flexibility, stress resilience, and interactions with the host environments.

Only 34 proteins exhibited opposite abundance patterns between *M. tuberculosis* and *M. bovis* under hypoxic and aerobic conditions (Table S2). Among these discordantly regulated proteins, four were involved in amino acid biosynthesis (ArgA, ArgH, AsD and AroD) and were exclusively enriched under hypoxia in *M. bovis*. The remaining proteins included six associated with lipid metabolism (EchA15, FabD, FadE1, FadE20, FadE4 and HsaG), primarily involved in lipid degradation, four related to energy metabolism and respiration (LpdC, NarL, NuoD and NuoF), and three involved in sulfur metabolism (AtsB, CysQ, MetB). However, except for the amino acid biosynthesis proteins, the other functional groups did not display a consistent species-specific pattern of accumulation, suggesting that their differential regulation was not systematically associated with either *M. tuberculosis* or *M. bovis*.

3. Conserved protein responses to hypoxic and aerobic growth in *M. bovis* strains

To identify potential protein markers of hypoxia in *M. bovis*, we compared the hypoxic proteome of AF2122/97 with that previously reported for the *M. bovis* NCTC10772 strain by our group [11]. This approach enabled the identification of proteins that were consistently associated with the hypoxic response across both strains (Table S3).

Under hypoxic conditions, STRING analysis of the shared proteins identified the Universal Stress Protein A family and nitroreductase-like proteins as the most strongly enriched functional groups (Fig. 3A). KEGG pathway enrichment analysis identified alanine, aspartate, and glutamate metabolism as the most significantly enriched metabolic pathway, pointing to a conserved adaptive response to oxygen deprivation

(Fig. 3A). Overall, 49 proteins showed increased abundance under hypoxia in both strains ($\text{padj} \leq 0.25$; Fig. 4).

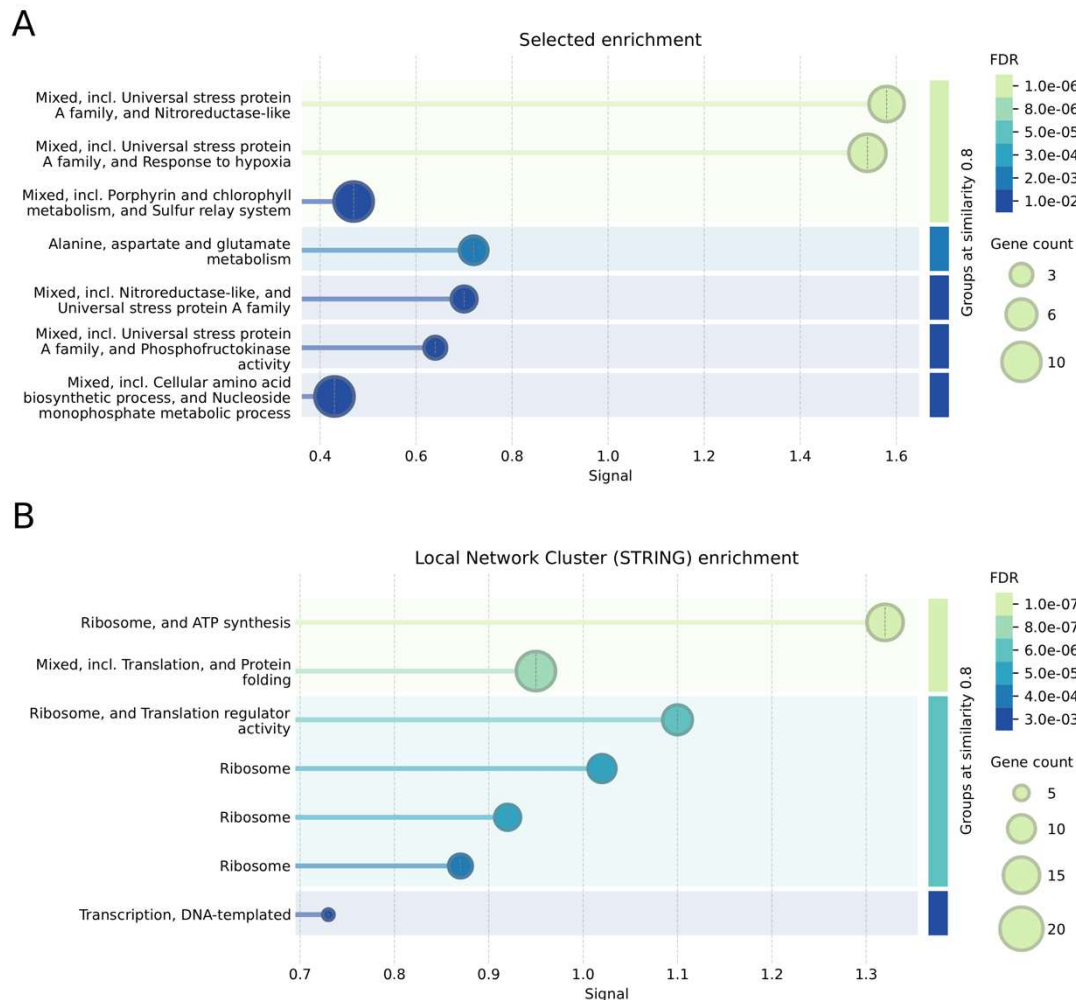


Figure 3. Functional enrichment analysis of shared proteins accumulated under aerobic and hypoxic conditions between *M. bovis* AF2122/97 and *M. bovis* NCTC10772. STRING local network cluster analysis was performed to identify functionally related proteins within each dataset. (A) Proteins shared between the two strains that accumulated under hypoxia. (B) Proteins shared between the two strains that accumulated under aeration. The x-axis represents the enrichment signal for each functional cluster, and circle size indicates the number of proteins associated with each cluster. Circle color represents the false discovery rate (FDR) of the enrichment analysis. Functional clusters are defined according to STRING database annotations.

Under aeration conditions, 64 proteins showed increased abundance in both strains ($\text{padj} \leq 0.25$; Table S3, Fig.4). STRING analysis identified enriched proteins involved in

ribosome structure and translation (Fig. 3B). RplI, RplL, RplE, RplO, RpmF, RpmE, RpsT, RpsP, Tig, Frr, NusG, GreA, RpoZ, and CarD form a functional network encompassing structural ribosomal proteins, translation-associated factors, molecular chaperones, and transcription regulators. Their coordinated increase in abundance is consistent enhanced protein synthesis and transcriptional activity during aerobic growth.

Several proteins that increased in abundance in both *M. bovis* strains were not induced under hypoxia in *M. tuberculosis*. These included LysX, RibF, FadE32, CydC, OppB, Rv1219c, PurU, and RocA. This pattern suggests the activation of additional metabolic adaptations during oxygen limitation.

RocA, a Δ^1 -pyrroline-5-carboxylate dehydrogenase, catalyzes the conversion of Δ^1 -pyrroline-5-carboxylate to glutamate, linking proline and arginine catabolism with central nitrogen metabolism. Consistent with this role, four additional proteins involved in alanine, aspartate, and glutamate metabolism (GlnA4, Gdh, PurQ, and CarB) showed an increased abundance under hypoxia in AF2122/97. Rv1188 (proline dehydrogenase), which catalyzes the preceding step in proline degradation, was also enriched. Together, these changes are consistent with increased engagement of the proline catabolic pathway and downstream glutamate metabolism during hypoxia. Given the central role of glutamate in nitrogen assimilation and biosynthetic reactions, the coordinated accumulation of these proteins suggests that *M. bovis* undergoes extensive remodeling of nitrogen metabolism as part of its adaptation to hypoxia.

FadE32, a fatty acid metabolism enzyme involved in mycolic acid biosynthesis, may support continued lipid utilization and cell envelope remodeling during hypoxia. RibF catalyzes the synthesis of the flavin cofactors FMN and FAD, and may therefore increase the availability of essential cofactors for numerous oxidoreductases involved in lipid catabolism and energy metabolism.

Consistent with a response to low oxygen availability, an increase in CydC, an ABC transporter required for the assembly and function of the high-affinity cytochrome bd oxidase, may enhance respiratory capacity under low-oxygen conditions. This is consistent with previous reports of increased expression of cytochrome bd oxidase genes during hypoxia in *M. tuberculosis* [4].

The increase of PurU, a protein that participates in tetrahydrofolate-dependent one-carbon metabolism, may contribute to nucleotide biosynthesis and metabolic flexibility

during nutrient limitation. Similarly, the peptide transporter OppB may facilitate peptide uptake as alternative source of carbon and nitrogen, whereas Rv1219c, previously associated with survival during prolonged stationary phase and persistence in vivo [29], may contribute to adaptation to prolonged hypoxic stress.

LysX, which catalyzes the lysinylation of phosphatidylglycerol, has also been associated with resistance to acidic stress [30].

The coordinated increase in RocA, ProDH (Rv1188), GlnA4, Gdh, PurQ, and CarB indicates that *M. bovis* strains (NCTC10772 and AF2122/97) supports a broader remodelling of nitrogen metabolism during hypoxia. These enzymes function sequentially or in interconnected pathways linking proline catabolism to glutamate production and glutamine-dependent biosynthesis [31], suggesting that proline may serve as an important metabolic substrate under oxygen-limited conditions. Because glutamate provides nitrogen for amino acid, nucleotide, and cofactor biosynthesis, increased activity of these pathways could help maintain essential anabolic processes despite the reduced metabolic activity associated with hypoxia. Moreover, the enrichment of both ProDH and RocA supports activation of the complete proline degradation pathway rather than isolated enzymatic changes. This coordinated response highlights the metabolic flexibility of *M. bovis* and suggests that remodeling of amino acid and nitrogen metabolism represents an additional adaptive strategy, complementing the well-established reliance on lipid metabolism during persistence under low-oxygen conditions.

Notably, this pattern was observed in both AF2122/97 and NCTC10772 but not in H37Rv, suggesting that *M. bovis* may rely more extensively on proline catabolism and nitrogen metabolic remodeling during hypoxic adaptation. This strain-associated difference may reflect distinct metabolic strategies that contribute to persistence under oxygen-limited conditions.

Proline degradation generates glutamate, which contributes to nitrogen homeostasis and can replenish the tricarboxylic acid (TCA) cycle through α -ketoglutarate. This metabolic adaptation may be particularly relevant to *M. bovis*, given its reduced glycolytic capacity associated with metabolic polymorphisms. A well-characterized example is the inactive pyruvate kinase PykA, which reduces glycolytic flux toward pyruvate [32]. However, whether increased proline catabolism compensates for this

metabolic constraint during hypoxia remains to be established and will require additional metabolic flux analyses.

4. Conserved protein responses to hypoxic and aerobic growth in *M. bovis* strains and *M. tuberculosis* H37Rv

Comparative analysis of proteins differentially expressed under aerobic and hypoxic conditions in *M. bovis* AF2122/97 (this study), *M. bovis* NCTC10772 (previous study), and *M. tuberculosis* H37Rv identified a limited number of hypoxia-associated proteins conserved across all three strains (Table 3, Fig. 4).

Table 3. Conserved proteins showing differential accumulation under hypoxic and aerobic conditions in *M. bovis* NCTC10772, *M. bovis* AF2122/97, and *M. tuberculosis* H37Rv

Growth Condition	Functional Category	Proteins
Hypoxia	Lipid metabolism and acyl-CoA generation	AccD1, BkdA, BkdB, CitE
	Stress response (DosRST regulon)	Rv1996, Rv2004c
	Molybdenum cofactor biosynthesis	MoeA2
	Predicted metabolic adaptation	Rv3161c
	Additional conserved hypoxia-associated proteins	GuaB3, Rv1194c, Rv3127, Rv3131, PknG
Aerobic	Virulence and host–pathogen interaction	CFP21, Apa, EspF, CspA
	Cell envelope biogenesis/remodeling	PonA2, MctB, Rv2129c
	Protein synthesis and turnover	RplI, RpoZ, PepA, PepD
	Central metabolism	HadB, Rv3376, Rv2772c
	Redox homeostasis	Rv0526
	Toxin–antitoxin systems	VapB43, VapB49
	Conserved hypothetical proteins	Rv3587c, Rv1072, TB15
	Transcriptional regulator	AlkX (Rv3249c)

Among the proteins that consistently showed increased abundance under hypoxia were AccD1, an acyltransferase involved in lipid biosynthesis, and BkdA, BkdB and CitE, which participate in BCAA catabolism and branched-chain lipid metabolism through the generation of acyl-CoA.

Proteins associated with molybdenum cofactor (MoCo) biosynthesis also showed increased abundance in both strains. MoeA2, which is involved in MoCo biosynthesis,

also showed higher levels under hypoxia. In addition, Rv2455c in *M. bovis* and Rv2454c in *M. tuberculosis* showed increased abundance. These proteins are encoded within a putative operon that also contains *mobA*, which encodes the probable molybdopterin-guanine dinucleotide biosynthesis protein A. Notably, Rv2455c and Rv2454c have previously been reported to increase in abundance in *M. tuberculosis* grown under hypoxic conditions using the Wayne model, supporting a conserved association between MoCo metabolism and adaptation to oxygen limitation [4].

By contrast, we did not detect increases in hypoxia-responsive MoCo biosynthesis proteins encoded by the *moaA1–moaD1* operon, which was previously shown to be induced during hypoxia by Levillain et al. [33]. This discrepancy may reflect variation in experimental conditions, the stage of hypoxic adaptation, or the relative contribution of distinct branches of the MoCo biosynthetic pathway. Thus, although MoCo metabolism appears to be a conserved feature of the mycobacterial response to oxygen limitation, the specific proteins contributing to this process may differ between strains and experimental settings.

Consistent with this possibility, Mycobrowser (<https://mycobrowser.epfl.ch/>) annotation indicates that at least 18 genes in *M. tuberculosis* and 13 genes in *M. bovis* reference strains encode proteins related to molybdenum metabolism. The breadth of this gene repertoire suggests that mycobacteria possess multiple components that could support MoCo-dependent metabolism under changing environmental conditions, although their specific contribution to hypoxic adaptation remains to be determined.

In addition, three DosRST-regulated proteins (Rv1996, Rv2004, and Rv3031) consistently showed higher abundance under hypoxia. Rv2004 contains a Z-toxin domain that has been associated in other bacterial systems with growth arrest, stress adaptation, and inhibition of cellular replication [34, 35].

Together, the conserved hypoxic response across the *M. bovis* and *M. tuberculosis* strains analyzed here and in our previous study was dominated by two functional modules: stress-response proteins, including members of the DosRST regulon, and proteins encoded by two adjacent operons responsible for BCAA catabolism. Whereas enrichment of stress-response proteins, particularly DosRST-regulated proteins, is a well-established feature of the mycobacterial response to hypoxia, the concurrent enrichment of BCAA catabolic proteins has not been previously reported. This finding

suggests that activation of this pathway may represent a conserved metabolic adaptation to oxygen limitation in both *M. tuberculosis* and *M. bovis*. BCAA degradation ultimately generates glutamate and intermediates that can contribute to nitrogen homeostasis and central carbon metabolism, while also providing precursors for lipid biosynthesis. This coordinated response may therefore increase metabolic flexibility during hypoxic adaptation.

Under aerobic conditions, *M. tuberculosis* and *M. bovis* strains showed increased levels of proteins associated with virulence, host–pathogen interaction, cell-envelope biosynthesis, and core cellular processes (Table 3). These included the secreted immunodominant antigens CFP21 and Apa, the ESX-1-associated proteins (EspF) [36], and the virulence-associated protein CspA, which showed the greatest increase in abundance and has been implicated in inhibition of phagosomal maturation (doi: 10.1080/08830185.2025.2531828). PonA2 and MctB, which have been linked to cell-envelope remodeling, were also enriched under aerobic conditions [37] [38].

Additional proteins represented functions related to protein synthesis and turnover (RplI, RpoZ, PepD, and PepA), nucleotide metabolism (Apt), redox homeostasis (Rv0526), toxin–antitoxin systems (VapB43 and VapB49) and transcriptional regulation. The latter included AlkX (Rv3249c), a transcriptional regulator that represses the *alkB-rubA-rubB* operon encoding an alkane hydroxylase/rubredoxin system [39]. Finally, several conserved hypothetical proteins (Rv1322A, Rv3587c, Rv1072, and TB15) consistently showed greater abundance under aerobic conditions across all three strains, which suggests that these proteins may contribute to conserved functions associated with aerobic growth.

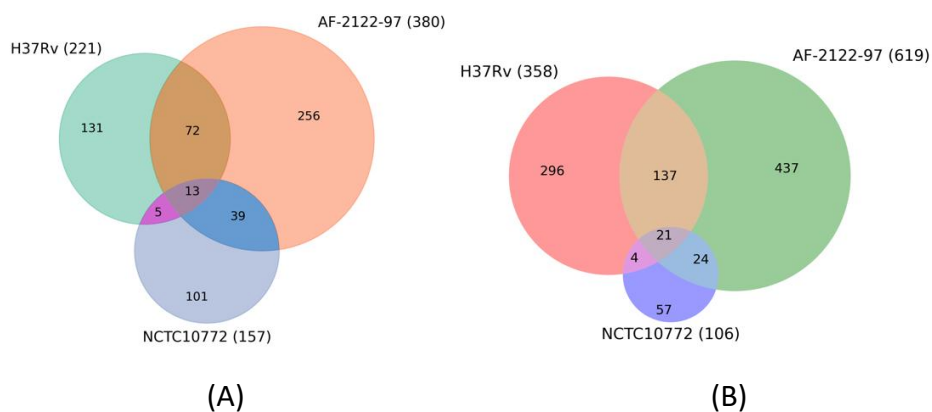


Figure 4. Overlap of proteins accumulating under hypoxia in *M. tuberculosis* H37Rv and *M. bovis* strains. Venn diagram showing the number of proteins identified as differentially accumulated under (A) hypoxia in *M. tuberculosis* H37Rv (221 proteins), *M. bovis* AF2122/97 (380 proteins), and *M. bovis* NCTC10772 (157 proteins); and after (B) aeration in *M. tuberculosis* H37Rv (358 proteins), *M. bovis* AF-2122-97 (619 proteins), and *M. bovis* NCTC10772 (106 proteins). Overlapping regions indicate proteins shared between two or three strains.

5. PDIM remodeling under hypoxia

The increased abundance of proteins involved in lipid metabolism under hypoxia compared with aerobic growth in both *M. bovis* and *M. tuberculosis* prompted us to examine whether these proteomic changes were accompanied by alterations in cell-envelope lipid composition. Among the hypoxia-associated proteins, enzymes involved in BCAA catabolism were consistently enriched under hypoxia in both the present study and our previous proteomic analysis. BCAA catabolism generates acyl-CoA, a key precursor for the biosynthesis of methyl-branched lipids. Given that phthiocerol dimycocerosate (PDIM) is a major methyl-branched lipid of the *M. tuberculosis* complex cell envelope, we compared PDIM profiles of H37Rv and AF2122/97 grown under hypoxic and aerobic conditions.

Both strains exhibited alterations in PDIM content under hypoxia compared with aeration (Fig. 5). In H37Rv, PDIM A levels decreased at all three hypoxia time points compared with aerobic growth. In AF2122/97, by contrast, both PDIM A and PDIM B were reduced at all hypoxia time points. Thus, despite the conserved enrichment of proteins involved in BCAA catabolism under hypoxia, the associated changes in PDIM composition differed between *M. tuberculosis* and *M. bovis*.

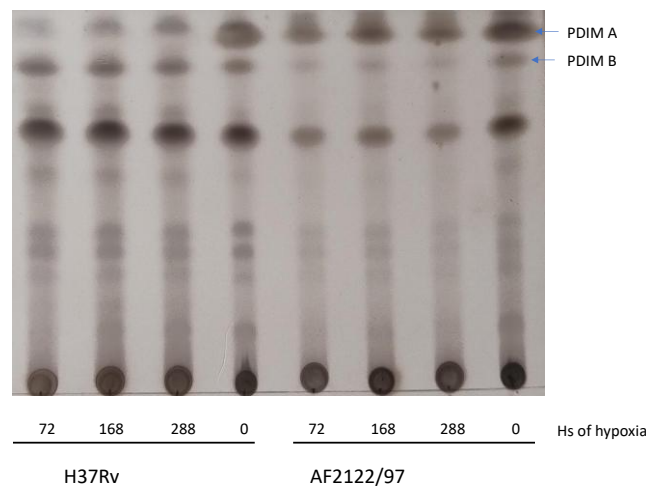


Figure 5. PDIM profiles of mycobacterial strains. Lipids were extracted from mycobacterial strains cultured under aerobic conditions (0 hs) or exposed to hypoxia (72, 168 and 288 hs). Lipid extracts were separated by TLC using the solvent system petroleum ether: ethyl acetate (98:2) as the solvent system to resolve PDIMs. Lipids were visualized by spraying with CuSO_4 followed by heating. These experiments represent one of two biological replicates with similar results.

These findings are consistent with the proteomic data and indicate that hypoxia is accompanied by cell-envelope lipid remodeling in both species, while suggesting that the specific lipid response is species dependent. Previous studies using the Wayne model have demonstrated substantial alterations in the mycolic acid and broader lipid profiles of *M. tuberculosis* in response to oxygen depletion. However, the reported changes have not been entirely consistent. An increase in specific mycolic acid species was observed under hypoxia in one study [40], whereas Galagan and collaborators [41] reported a depletion of several mycolic acid species under hypoxic conditions. Despite these contrasting findings, both studies support the concept of extensive lipid remodeling as an important component of the *M. tuberculosis* response to oxygen limitation. The depletion of specific lipid species may reflect their mobilization as carbon and energy sources through fatty acid degradation and β -oxidation, a metabolic strategy that has been proposed to contribute to mycobacterial persistence during hypoxia and dormancy. In this context, the accumulation of other lipid species may represent compensatory remodeling of the cell envelope to maintain membrane integrity and

adapt to the altered physiological state. Thus, the apparently divergent lipid profiles observed across studies may reflect the dynamic reorganization of lipid metabolism during adaptation to hypoxic stress, including a balance between lipid synthesis, remodeling, and degradation.

6. Effect hypoxia on the viability of *M. tuberculosis* and *M. bovis*

The ability of *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv to recover growth after prolonged oxygen deprivation was evaluated by serially diluting cultures exposed to hypoxia for different periods and subsequently plating the dilutions on solid medium for CFU determination. H37Rv maintained viability throughout the hypoxic exposure, with no significant differences in CFU counts between early and prolonged hypoxia (Fig. 6). In contrast, AF2122/97 showed a marked reduction in viable bacterial counts following exposure to hypoxia, which progressively more pronounced with increasing duration of oxygen deprivation.

AF2122/97 cultures also showed a decrease in OD during hypoxic incubation. However, the magnitude of this change did not parallel the pronounced decline in CFU counts. This divergence between biomass and viability is consistent with a hypoxia-induced transition to a non-replicating or poorly culturable state.

To assess whether this phenotype is conserved among other *M. bovis* isolates, we quantified CFUs in *M. bovis* NCTC10772 following prolonged hypoxia and subsequent reaeration. Unlike AF2122/97, NCTC10772 showed a reduction in viability comparable to that observed in *M. tuberculosis* H37Rv (Fig. 6). Thus, impaired recovery following reaeration is not a general feature of *M. bovis* but rather a strain-specific characteristic of AF2122/97.

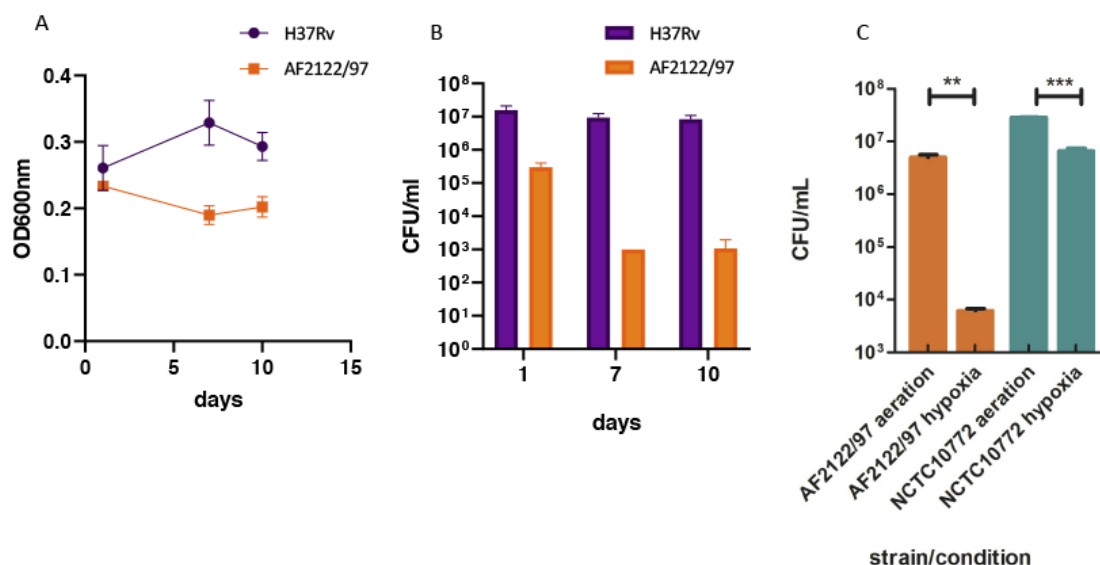


Figure 6. Growth of mycobacterial strains under the Wayne hypoxia model. Mycobacterial strains were grown to exponential phase ($OD_{600} = 0.5$), diluted to $OD_{600} = 0.45$, and plated for CFU determination under aerated conditions. For hypoxia experiments, cultures were further diluted to $OD_{600} = 0.05$ and incubated under the Wayne model. OD_{600} was determined at different time points during hypoxic incubation (A), and CFUs were determined at the corresponding time points (B) or after 10 days of hypoxic incubation (C). Measurements were performed in triplicate. Data are presented as mean $\pm$ SD. These experiments represent one of three biological replicates with similar results. Data were analyzed with two-way ANOVA and Turkey's multiple comparisons test (** $p < 0.01$, *** $p < 0.001$).

Conclusions

In this study, we identified proteins that accumulated during hypoxia and subsequent aeration in the reference strains *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv. A major finding was the identification of pathways involved in acyl-CoA generation as a conserved response activated under hypoxia in both species. Proteins associated with lipid metabolism and stress responses, particularly members of the DosRST regulon, were prominent features of hypoxic adaptation in both strains. Notably, more DosRST- and EHR-regulated proteins showed increased levels in *M. bovis* than in *M. tuberculosis*. This difference should, however, be interpreted with caution, as it may partially reflect the lower number of differentially accumulated proteins identified in H37Rv, potentially due to technical limitations.

Lipid metabolism and remodeling were central features of the hypoxic response in both species. These findings support the notion that pathogenic mycobacteria rely on lipid utilization to maintain energy homeostasis and redox balance under oxygen-limited conditions. Cell-envelope remodeling may also contribute to hypoxic adaptation by preserving cellular integrity and promoting long-term survival under adverse conditions. Consistent with the changes in proteins involved in lipid metabolism and cell envelope remodeling, hypoxia altered the levels of PDIM A and PDIM B in both species. However, the distinct patterns of PDIM modulation in *M. tuberculosis* and *M. bovis* suggest that these pathogens may use species-specific lipid remodeling strategies during adaptation to hypoxia. Further studies are required to elucidate the mechanisms underlying PDIM remodeling and determine its functional contribution to persist under oxygen limitation. Both species also showed accumulation of proteins involved in molybdenum metabolism, suggesting a role for molybdenum-dependent processes in hypoxic adaptation. By contrast, changes in nitrogen metabolism were a prominent feature of the *M. bovis* response, pointing to differences in metabolic adaptation between *M. bovis* and *M. tuberculosis* during oxygen deprivation.

A limitation of this study is that the analysis was restricted to the cellular proteome and did not capture changes in the secreted proteome. Comprehensive analysis of the secretome, together with proteomic profiling at multiple stages of hypoxia and re-aeration, will be necessary to acquire a more complete understanding of the mechanisms used by *M. tuberculosis* and *M. bovis* to adapt to hypoxic stress. Such studies could clarify species-specific differences underlying their distinct physiological responses and inform the use of these organisms as models of latency and dormancy.

Supplementary material

Table S1. Total proteins detected in *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 grown under hypoxia and aeration

Table S2. Proteins accumulated in *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 under hypoxia and aeration ($\log_2FC \geq |2|$, $p_{adj} \leq 0.25$)

Table S3. Shared proteins accumulated under hypoxia and aeration between *M. tuberculosis* H37Rv and *M. bovis* AF2122/97

Table S4. Shared proteins accumulated under hypoxia and aeration between *M. bovis* AF2122/97 and *M. bovis* NCTC10772.

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