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Suppression of Eis and expression of Wag31 and GroES in *Mycobacterium tuberculosis* cytosol under anaerobic culture conditions†

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A major impediment in chemotherapy of Tuberculosis (TB) is the persistence of *M. tuberculosis* in a latent or dormant state, possibly perpetuated by paucity of oxygen within the lung granuloma. Proteome analysis of the anaerobically persisting microbe could therefore provide novel targets for drugs against latent TB infection (LTBI). An Indian clinical isolate of *M. tuberculosis* was cultured under aerobic and anaerobic conditions following Wayne's hypoxia model and its cytosolic proteins were resolved by two-dimensional gel electrophoresis (2DE). Peptide mass fingerprinting of 32 differentially expressed spots using MALDI TOF-TOF MS-MS resulted in identification of 23 proteins. Under the anaerobic culture conditions, expression of 12 of these proteins was highly suppressed (>2 fold reduction in spot volumes), with 4 of them (GrpE, CanB, MoxR1 and Eis) appearing as completely suppressed since corresponding spots were not detectable in the anaerobic sample. On the other hand, 4 proteins were highly expressed, with two of them (Wag31 and GroES) being uniquely expressed under anaerobic conditions. Suppression of Eis could make the anaerobically persisting bacilli susceptible to the aminoglycoside antibiotics which are known to be acetylated and inactivated by Eis. Although all 4 over-expressed proteins can be considered as putative drug targets for LTBI, Wag31 appears particularly interesting in view of its role in the cell wall biogenesis.

Keywords: Anaerobic persistence, Eis, GroES, *Mycobacterium tuberculosis*, LTBI, TB, Wag31

Over a third of world's population harbors 'latent' tuberculosis infection (LTBI) and, from this vast pool of potential TB cases, 5-10% develop active disease during their lifetime[1]. Primary infection with *Mycobacterium tuberculosis* typically leads to replication of bacilli inside alveolar macrophages until an effective immune response restricts them to the granulomas. Though the environment within a granuloma is considered as hostile, the bacilli can survive in it for long durations[2]. These latent (or dormant) bacilli are unresponsive to currently available chemotherapy and remain a potential source of activation of the disease[1]. Elucidation of *M. tuberculosis* genes and proteins which are specifically modulated during latency is therefore considered essential for identification of potential targets for new drugs against LTBI[3].

Relative avascularity and poor permeability of the granuloma strongly suggest that scarcity of oxygen is a major factor that drives *M. tuberculosis* bacilli into latency[3,4]. Wayne[5] demonstrated that bacilli that settle through a 'self-generated' oxygen depletion gradient undergo an orderly metabolic shift-down. Accumulating at the bottom of culture tube, they enter a homogeneous physiologic state of dormancy and exhibit a 'synchronous' replication cycle upon re-exposure to air[6]. In this state of 'non-replicating persistence (NRP)' most RNA and protein synthesis stops, though there is an enhanced production of isocitrate lyase (Icl), glycine dehydrogenase, Acr and few other proteins[7,8]. The bacilli become resistant to many antibiotics, including isonizaid and rifampicin, but show susceptibility to metronidazole[9]. A subsequent study using knockout of Icl has confirmed the importance of glyoxylate pathway in persistence of *M. tuberculosis*[10].

In a proteomic study based on Wayne's model of LTBI, Cho *et al.*[11] subjected *M. tuberculosis* cell lysates to Isotope Coded Affinity Tagging (ICAT) in order to identify proteins which were expressed differentially during NRP stages. Nonetheless, ICAT technology has some inherent limitations. It requires presence of cysteine in the protein whereas over 18% of *M. tuberculosis* proteins lack cysteine residues[11]. Another evaluation has shown that the ICAT method under-represents small proteins and is biased towards

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detecting the acidic proteins[12]. Two-dimensional gel electrophoresis (2DE), which is a widely used method for proteome analyses[13], also complements ICAT. In a study of *M. tuberculosis* proteome, 108 proteins were identified by 2DE and 280 by ICAT. However, only 27 were common to both[14]. In another study of rat brain endothelial cells 28 proteins were identified by 2DE and 138 by ICAT, with only 15 of them showing a shared presence[15]. In this particular study, although ICAT identified a larger number of proteins, some important protein changes were identified only by 2DE.

In the present study, 2DE based proteomics have been used to identify proteins which were differentially expressed during anaerobic persistence in an Indian clinical isolate of *M. tuberculosis*. For this, the stationary culture model described by Wayne[6,9] has been adopted. Another important feature of this study is that attention has been focused on proteins of the cytosol rather than 'whole cell lysate'[11,16,17] so as to work with a less complex and more soluble sample, which could also facilitate the detection of less abundant proteins[13].

Materials and Methods

Mycobacterium tuberculosis—A drug-sensitive clinical isolate of *M. tuberculosis*, obtained from ICMR-Tuberculosis Research Centre, (now National Institute for Research in Tuberculosis) Chennai, India was used in this study. The seed culture was maintained on Löwenstein-Jensen medium.

Cultures under aerobic and anaerobic conditions—Cultures were set according to the method described by Wayne and Sramek[6,9]. In brief, aliquots of Middlebrook 7H9 broth (supplemented with albumin-dextrose-catalase) were dispensed in screw-capped tubes and inoculated with *M. tuberculosis*. For aerobic cultures, tubes were capped loosely and incubated (37 °C, 2 weeks) with constant shaking in a rotary shaker-incubator. At the time of harvesting, these cultures attained an OD_{580} of ~ 0.5. For anaerobic cultures, tubes were tightly capped and incubated in upright position without any disturbance. Attainment of anaerobic conditions was confirmed by de-colorization of methylene blue (2.5 µg/ mL), added to one of the culture tubes. At the end of incubation period (4 weeks), the supernatant was carefully removed and dormant cells settled at the bottom of the tube were collected by centrifugation. The harvested bacilli, upon reconstitution with fresh medium, showed a typical 'synchronous' multiplication pattern[6]. Three biological replicates of each of the aerobic and anaerobic bacilli were prepared in this manner and subjected to proteome analyses.

Cytosolic proteins—The cytosolic proteins of *M. tuberculosis*, grown under aerobic and anaerobic conditions, were isolated by using a previously described method[18]. In brief, the bacilli were washed and re-suspended in Tris buffer containing protease inhibitors. The bacterial suspensions were probe-sonicated and centrifuged (150,000 *g*) to remove the sediment comprising unbroken cells and cell envelope debris. The supernatant (cytosol) was collected and constituent proteins were precipitated with tri-chloro acetic acid (15% final concentration). The precipitates were recovered by centrifugation and washed with chilled acetone. Air-dried proteins were reconstituted in water and protein estimations were done using a modified Lowry method[19]. Required aliquots were lyophilized and stored at −20 °C.

2DE—A previously described protocol[18] was used with some modifications. Aliquots of 200 µg lyophilized cytosolic proteins were dissolved, by repeated pipeting through a micropipette, in 200 µL sample solubilization medium (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% carrier ampholytes, 1% dithiothreitol, 10% isopropanal and 5% glycerol). After centrifugation (15,000 *g*, to remove any insoluble matter), the solubilized proteins were applied (150 µL/strip) to immobilized *p*H gradient (IPG) strips (7 cm, *p*H 4-7; Bio-Rad Laboratories, USA) in a reswelling tray and left overnight for rehydration. Isoelectric focusing (IEF) was performed with a programmable power supply (Investigtor-5000; Genomic Solutions, USA) using following parameters: maximum voltage, 3000; maximum current, 50 µA/ strip; total volt hour, 10000 and total run time, 8 h. After IEF, the IPG strips were equilibrated (10 min each) sequentially in solutions 'A' (0.05 M Tris-HCl, *p*H 8.8 containing 6 M urea, 30% glycerol, 2% SDS, and 1% dithiothreitol) and 'B' (solution 'A' without dithiothreitol, but with 4% iodoacetamide and 0.005% bromophenol blue). The strips were later loaded on top of SDS-polyacrylamide gel slabs (12% gel, 1 mm thick) and electrophoresis was performed at a constant current (15 mA). The gels were stained with Coomassie Brilliant Blue R250.

Gel imaging and image analysis—Gel imaging was performed on a Pro Express 2D Proteomic Imaging System (Perkin Elmer, USA). Phoretix-2D software

(Perkin Elmer, USA) was used for image analysis, spot detection and determination of spot volumes. Spots from *M. tuberculosis* cultured under aerobic and anaerobic conditions were matched and differences in spot volumes were considered valid only if seen in at least two of the biological replicates. Spots of interest were manually excised and processed for protein identification.

Protein identification—Proteins (in gel plugs) were processed according to standard techniques[20]. In brief, samples were washed, in-gel reduced, S-alkylated, and digested overnight with trypsin. The extracted peptides were vacuum dried, resolubilized and desalted with ZipTips (Millipore, USA). Matrix-assisted laser desorption-ionization (MALDI) time-of-flight (TOF)-TOF mass spectrometry (MS)-MS was performed on a 4800 MALDI TOF/TOF TM analyzer (Applied Biosystems/MDS SCIEX, Proteomics International, Perth) using a previously described procedure[21]. In brief, peptide samples were mixed with matrix solution (α-cyano 4-hydroxy cinnamic acid), spotted on a 384-well Opti-TOF stainless steel plate and analysed using a first run of standard TOF MS. The system was set to perform a second run of MS/MS focused on 15 most intense peaks of the first MS (excluding peaks known to be trypsin). The laser was set to fire 400 times per spot in MS mode and 2000 times per spot in MS/MS mode. Laser intensity was 2800 J (MS) and 3900 J (MS/MS). A mass range of 400–4000 amu with a focus mass of 2100 amu was used. Mass spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) and the non-redundant sequence database from NCBI (http://www.matrixscience.com/help/seq_db_setup_nr.html), with taxonomy set to '*Mycobacterium tuberculosis* complex'. Protein identifications were based on probability-based Molecular Weight Search (MOWSE) scoring algorithm, using a confidence level of 95% ($P < 0.05$).

Bioinformatic analyses—Data mining for identified proteins was performed from the databases of Tuberculist (http://tuberculist.epfl.ch) and Uniprot (http://www.uniprot.org). Information on previously identified proteins was also obtained from the same databases as well as published literature.

Results

Differential expression of M. tuberculosis proteins—The applied 2DE protocol provided a clear resolution of the cytosolic proteins of bacilli cultured under aerobic or anaerobic conditions (Fig. 1, A and B). Reproducibility of the resolution patterns was confirmed by performing 2DE with biological replicates. Comparative evaluation of the two electrophoretograms revealed differential expression of 32 protein spots. Of these, volumes of 21spots (H-1 to H-21; Fig. 1A) were reduced and those of 11 spots (H-22 to H-32; Fig. 2B) were enhanced under anaerobic culture conditions. An important observation was that 7 of these spots (H-2, 11-13, 19-21) appeared

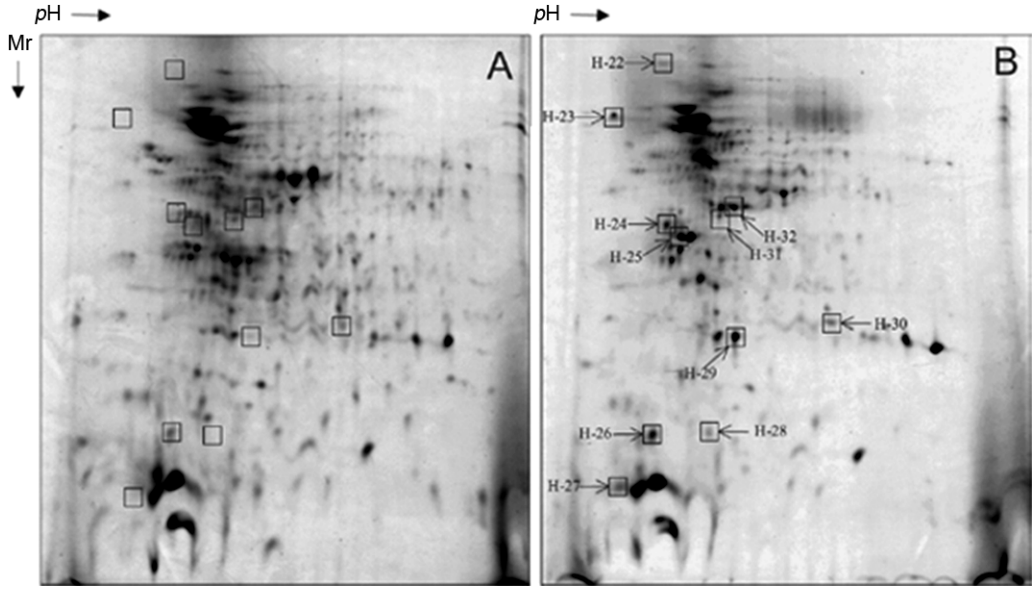

Fig. 1—Resolution by 2DE of the cytosolic proteins of *M. tuberculosis* (clinical isolate) cultured under aerobic (A) and anaerobic (B) conditions. 21 protein spots (H-1 to 21, A), which appeared either completely (H-2, 11-13, 19-21) or partially suppressed under anaerobic conditions (corresponding loci shown in B), were picked and processed for identification.

unique to the aerobic sample and 5 others (H-22, 23, 25, 27 and 28) were unique to the anaerobic sample.

Analysis of the 32 differentially expressed spots by MALDI TOF-TOF MS-MS led to identification of constituent proteins in 25 spots. Protein in the remaining 7 spots (H-7, 8, 11, 12, 22, 28 and 29) could not be identified due to insignificant or ambiguous peptide mass matches (MOWSE scores). Further, a couple of spots in each gel (H-19 and 21, Fig. 1A; H-23 and 25, Fig. 2B), which were most probably separated due to post-translational modifications, represented the same protein. For instance, Wag31 (represented by spots H-23 and H-25) is known to exist as self-interacting, membrane-associated oligomers[22]. Thus, a total of 23 proteins were identified.

Identity of the suppressed proteins—Twelve proteins showed >2 fold reduction in spot volumes under anaerobic conditions (Table 1). More importantly, 4 of them (GrpE, CanB, Eis and MoxR1) appeared to be completely suppressed since corresponding spots were not detectable in the anaerobic cytosol. The remaining 8 proteins were: Rv3699 (conserved protein, CP), Rv2185c (CP), GreA, Rv2140c (CP), Rv1404, Rv0148, Rv0831c (CP) and Tuf. Four other proteins that were picked from the aerobic cytosol (H-1, 4, 14 and 16, Fig. 1A) showed < 2 fold change in spot volumes, which was not considered as significant (Table 3).

Identity of the over-expressed proteins—Four proteins showed >2 fold enhancement in spot volumes under anaerobic conditions (Table 2). Two of them

Table 1—The cytosolic proteins of *M. tuberculosis* which were either completely suppressed or reduced > 2 fold under anaerobic culture conditions

Spot No.*	Gene	Protein	MOWSE§	pI	Mr (kDa)	Change in spot volume
H-2	Rv0351	Hsp70 cofactor, GrpE	73/29	4.39	24.53	Complete Suppression$
H-13	Rv3588c	Carbonic anhydrase, CanB	91/28	5.60	21.79	Complete Suppression
H-19, H-21	Rv2416c	Enhanced intracellular survival protein, Eis	111/28, 109/28	5.97	44.41	Complete Suppression
H-20	Rv1479	Probable transcriptional regulatory protein, MoxR1	80/29	5.96	40.76	Complete Suppression
H-3	Rv3699	Conserved Protein	146/28	4.69	25.25	(-) 2.209$$
H-5	Rv2185c	Conserved Protein	406/28	4.75	16.32	(-) 2.057
H-6	Rv1080c	Transcription elongation factor, GreA	197/28	4.90	17.80	(-) 2.039
H-9	Rv2140c	Conserved Protein	256/28	5.41	18.63	(-) 2.293
H-10	Rv1404	Probable transcriptional regulatory protein	257/29	5.75	17.52	(-) 2.041
H-15	Rv0148	Probable short chain type dehydrogenase	635/28	5.26	29.77	(-) 2.413
H-17	Rv0831c	Conserved Protein	569/29	5.09	30.18	(-) 2.671
H-18	Rv0685	Elongation factor Tu, Tuf	1208/28	5.28	43.59	(-) 2.576

* All spots were picked from the aerobic sample (Fig. 1A).

§ Molecular Weight Search score reported by Mascot, expressed as actual score/minimum required for attaining $P < 0.05$.

$ Not detected in the anaerobic sample.

$$ Fold decrease in spot volume as compared with the aerobic sample.

Table 2—The cytosolic proteins of *M. tuberculosis* which were either uniquely expressed or enhanced > 2 fold under anaerobic conditions

Spot No.*	Gene	Protein	MOWSE§	pI	Mr (kDa)	Change in spot volume
H-23, H-25	Rv2145c	Ag84, Wag31	91/29, 103/28	4.80	28.27	Unique Expression$
H-27	Rv3418c	10 kDa chaperonin, GroES	373/28	4.62	10.80	Unique Expression
H-24	Rv0934	Periplasmic phosphate-binding lipoprotein, PstS1	345/28	5.14	38.24	(+) 5.091$$
H-32	Rv1436	Glyceraldehyde-3-phosphate dehydrogenase, Gap	44/28	5.19	35.95	(+) 2.251

*All spots were picked from the anaerobic sample (Fig. 2B).

§ Molecular Weight Search score reported by Mascot, expressed as actual score/minimum required for attaining $P < 0.05$.

$ Not detected in the aerobic sample.

$$ Fold increase in spot volume as compared with the aerobic sample.

(Wag31 and GroES) appeared as uniquely expressed, since corresponding spots were not detectable in the aerobic sample. The other two proteins were PstS1 and Gap. Spot volumes of the remaining 3 proteins (H-26, 30, 31, Fig. 2B) that were picked from the anaerobic sample showed <2 fold change (Table 3).

Discussion

To our knowledge, this is the first report on 2DE based proteomics using Wayne's model of anaerobic persistence in *M. tuberculosis*. A previous study using this model applied ICAT based proteomics[11] which shows major differences with 2DE in terms of protein coverage[12,14,15]. For instance, monitoring the expression levels of GroES, which was one of the uniquely expressed proteins in this study, could have not been possible by ICAT as it lacks cysteine[14]. The other 2DE based proteomic studies on *M. tuberculosis* under oxygen limiting conditions[16,17] did not replicate Wayne's model. Rosenkrands *et al.*[16] performed short-term (20 h) cultures of *M. tuberculosis* in tightly capped tubes as well as under predefined oxygen tensions (1, 5 and 20%) to identify 7 over expressed proteins. Starck *et al.*[17] also cultured *M. tuberculosis* in a defined hypoxic atmosphere (85% N_2, 10% H_2, 5% CO_2) and identified 16 over expressed proteins. Due to the difference in culture conditions[23], only 2 proteins (Acr and Ald) were common in both studies. Apparently for the same reason, we could also detect only 2 proteins (Tuf and Rv2185c) from the latter study[17]. Even so, their expression was

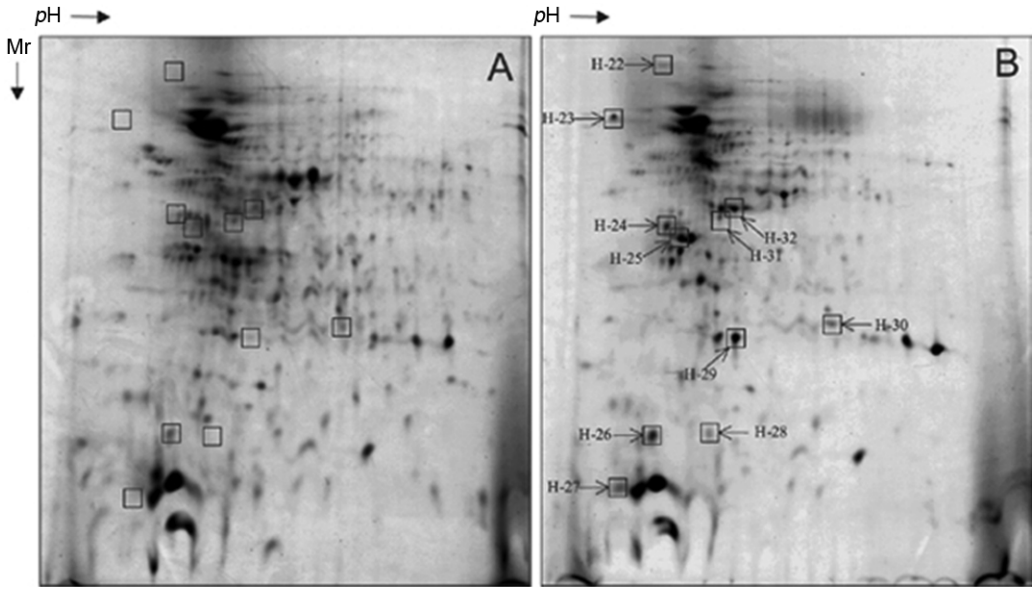

Fig. 2—Resolution by 2DE of the cytosolic proteins of *M. tuberculosis* (clinical isolate) cultured under aerobic (A) and anaerobic (B) conditions. 11 protein spots (H-22 to H-32, B) which appeared either uniquely (H-22, 23, 25, 27 and 28) or highly expressed in the anaerobic sample were picked for identification. Corresponding loci are marked in A.

Table 3—*M. tuberculosis* proteins which showed < 2 fold change under anaerobic conditions

Spot No.*	Gene	Protein	MOWSE§	pI	Mr (kDa)	Change in spot volume$
H-1	Rv2462c	Trigger factor, Tig	1094/28	4.43	50.61	(-) 1.081
H-4	Rv2557	Conserved Protein	101/28	4.80	24.29	(-) 1.171
H-14	Rv3246c	Two component sensory trandsuction transcriptional regulatory protein, MtrA	136/27	5.50	24.93	(-) 1.170
H-16	Rv2889c	Elongation factor TS-9, Tsf	272/28	5.26	28.75	(-) 1.203
H-26	Rv0652	50S ribosomal protein, RplL L7/L12	381/28	4.59	13.44	(+) 1.542
H-30	Rv0632c	Enoyl CoA hydratase, EchA3	253/28	5.52	24.34	(+) 1.545
H-31	Rv3001	Keto acid reductoisomerase, IlvC	234/28	5.03	36.09	(+) 1.124

*Spots H-1 to H-16 were from aerobic sample (Fig. 1A), and H-26 to H-31 were from the anaerobic sample (Fig. 2B).

§ Molecular Weight Search score reported by Mascot, expressed as actual score/minimum required for attaining $P < 0.05$.

$ Fold decrease (-) or increase (+) in spot volumes, compared with the aerobic sample.

reduced. While most earlier studies[11,16,17] have analysed the 'whole cell lysate' of *M. tuberculosis*, we chose to study the cytosol so as to work with a less complex sample which could also improve the detection of 'low abundance' proteins[13]. This approach may have helped us visualise certain proteins which were hitherto not associated with anaerobic persistence. However, it was also the reason behind our inability to detect the Acr homolog which is highly expressed by the microbe under anaerobic conditions[8]. As Acr is located in the cell envelope[8], it was not detectable in the cytosol.

As a strategy for survival during latency, the microbe is likely to curtail its metabolic activity, exploit alternate energy sources and develop mechanisms that stabilize its cell architecture. Thus, several metabolic pathways, including protein synthesis in general, are expected to be down regulated[24]. This is also evident from the present results. Four proteins, which were completely (MoxR1) or highly (GreA, Rv1404 and Tuf) suppressed, are involved in protein synthesis (transcription/translation). Three other proteins—CanB, GrpE and Eis also appeared as completely suppressed. Carbonic anhydrases are metalloenzymes involved in respiration[25] and it explains down regulation of CanB under anaerobic conditions. GrpE is a part of the dnaK-grpE-dnaJ-hspR operon. As a co-chaperone, it enables recycling of DnaK (Hsp70)[26]. However, the present results suggest that GrpE may not be essential for anaerobic persistence. In an earlier study[27], its levels were also found to be decreased during starvation. The biological role of *M. tuberculosis* Eis has been a subject of major interest[28]. Eis homologues are found in a variety of pathogens which have developed resistance to aminoglycosides (AG). Recently an unprecedented multiacetylation capability of Eis, that inactivates AG antibiotics, has been discovered[29]. It would be interesting to learn if the suppression of Eis can result in enhanced susceptibility of the dormant bacilli to such antibiotics.

The proteins whose production was enhanced despite the metabolic shift down could be considered as essential for survival of *M. tuberculosis* under anaerobic conditions. Two proteins (Wag31 and GroES) were uniquely, and two others (PstS1 and Gap) were highly (>2 fold) expressed. Wag31 is an essential protein as its depletion causes abnormal bacterial morphology due to a defect in polar peptidoglycan synthesis[30]. A fairly high capacity for peptidoglycan biosynthesis is also maintained by bacteria in their latent phase[31]. Though, Wag31 remains non- or lowly- phosphorylated during latency, it can still be recruited to the cell poles leading to polar peptidoglycan synthesis[22]. Wag31 also interacts with PBP3 (or FtsI), which is essential for the synthesis of septal peptidoglycan[32]. The stringent response of *M. tuberculosis* is coordinated by Rel and it has been shown that Wag31 is up-regulated in *M. tuberculosis* in a Rel-dependent manner[33]. Thus, a drug that targets Wag31 is likely to make the latent *M. tuberculosis* more vulnerable to chemotherapy.

Interaction between the chaperonin GroEL and its co-chaperonin GroES is necessary for folding of a variety of polypeptides in an ATP-dependent manner. About 10-15% of all newly synthesized polypeptide chains interact with GroEL-GroES under normal conditions and this number rises to >30% upon exposure to stress[34]. It seems interesting that the anaerobically persisting *M. tuberculosis*, while jettisoning one of its co-chaperones (GrpE, discussed above), has chosen to not only retain but also overproduce the other (GroES). Evidently, GroES possesses some additional properties which could help *M. tuberculosis* survive under anaerobic conditions. A study[35] has demonstrated that GroES can act as a transcription regulator hence can modulate gene expression, particularly in conditions like stress.

The membrane-associated phosphate-specific transporter complex is composed of four different proteins: Pst-S, A, B and C. In the *M. tuberculosis* genome, different gene clusters encode three PstS proteins. It has been shown that the multiplication of pstS1 and pstS2 knockouts in the mouse lung was sluggish and the bacteria were eliminated faster compared to the parental strain, suggesting that *M. tuberculosis* encounters limiting phosphate concentrations *in vivo*[36]. These results have led to the hypothesis that PstS transporters are virulence factors of *M. tuberculosis*. However, a more direct role of PstS in maintenance of virulence during latency is yet to be elucidated.

Glyceraldehyde 3-phosphate dehydrogenase (Gap) is a key enzyme in intermediary metabolism and operates at the sixth step in glycolysis, during which glucose is converted to pyruvate with the net generation of two ATP molecules. Pyruvate can be anaerobically metabolized to ethanol by the enzyme

alcohol dehydrogenase and NADH. This reaction regenerates a pool of NAD allowing glycolysis to occur under anaerobic conditions[37]. Thus, up-regulation of Gap may be necessary to satisfy the energy demands of *M. tuberculosis* during latency. Gap was also among the proteins that were over expressed in the stationary cultures of BCG[38].

In conclusion, a decline in the expression levels of certain *M. tuberculosis* proteins under anaerobic conditions may generally be viewed as the consequence of a subdued metabolic activity of the microbe. Nonetheless, a 'near-complete' suppression of Eis could create an opportunity for the use of aminoglycoside antibiotics in the treatment of latent TB. On the other hand, each of the four proteins that were uniquely or highly expressed under anaerobic conditions qualify as putative targets for new drugs against latent TB. In particular, there is a strong case for Wag31 since, even if not replicating, the bacilli would need to keep repairing, renewing and strengthening their most vital component- the cell wall.

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Disclosure

None of the authors has any conflict of interest associated with this study.

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