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# Evaluation of the role of sigma B in *Mycobacterium smegmatis* ☆

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**Abstract**

The alternate sigma factor, *sigB*, is known to play a crucial role in maintaining the stationary phase in mycobacteria. In this communication, we have studied the proteomics of *Mycobacterium smegmatis* $mc^2155$ and its two derivatives, one of which has a disrupted *sigB* gene and the other, PMVSigB, which contains a multicopy plasmid containing *sigB*. We have identified by two-dimensional gel analyses, several proteins that are over-expressed in PMVSigB compared to $mc^2155$. These proteins are either stress proteins or participate actively in different metabolic pathways of the organisms. On the other hand, when *sigB* deleted mycobacteria were grown until the stationary phase and its two-dimensional protein profile was compared to that of $mc^2155$, few DNA binding proteins were found to be up-regulated. We have shown recently that upon over-expressing *sigB*, the cell surface glycopeptidolipids of *M. smegmatis* are hyperglycosylated, a situation similar to what was observed for nutritionally starved bacteria. Gene expression profile through quantitative PCR presented here identified a Rhamnosyltransferase responsible for this hyperglycosylation.


*Keywords:* 2-D PAGE; *Mycobacterium smegmatis*; *sigB*; Glycosyltransferase

*Mycobacterium smegmatis*, a gram-positive soil bacterium, is often exposed to nutrient scarcity and other environmental offensives. The ability of the organism to survive in this milieu solely depends on sensing the changes and responding to them through complex adaptational networks. Bacteria possess several sigma factors which upon binding to the core RNA polymerase regulate expression of different set of genes as per the need of the organism [1]. Regulation of gene expression by alternative sigma factors, anti-sigma factors, and anti-anti-sigma factors in response to environmental condition is essential for bacterial survival, which has been elegantly demonstrated in the case of *Bacillus subtilis* [2,3]. Like any other bacteria, mycobacteria also have a group of alternative sigma factors and two component response regulators, which get stimulated under different kinds of stresses.

The alternative sigma factor *sigB* has considerable homology among different species and therefore appears to perform similar functions [4]. It is known to be a stress-specific sigma factor, which regulates gene expression, upon acid stress in *Listeria monocytogenes* [5], during heat shock and general stress in non-growing cells of *Staphylococcus aureus* [6,7]. The *sigB* null mutants of *S. aureus* were also sensitive to $H_2O_2$ [8]. In *Bacillus subtilis*, SigB directs the transcription of more than 200 genes [9], and the transcription of at least 42 general stress proteins is dependent on the alternative sigma factor SigB [10]. The actinomycetes *Streptomyces coelicolor*, closely related to the genus mycobacteria, has as many as nine SigB paralogues to induce different independent set of genes [11]. In the virulent mycobacteria, the stress responsive sigma factor, which is reported to be most similar to the SigB of other eubacteria, is SigF, having 30% identity to the *B. subtilis* SigB [12]. However, the function of mycobacterial SigB remained mostly unexplained.

☆ *Abbreviations:* 2-D, two-dimensional; IPG, immobilized pH gradient; LIFT, laser induced fragmentation technique; MALDI-TOF-MS, matrix assisted laser desorption ionization time of flight mass spectrometry; MS–MS, tandem mass spectrometry; TCA, tri-carboxylic acid; TFA, trifluoroacetic acid.

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The mycobacterial *sigB*, which is closely related to the major, indispensable sigma factor *sigA* [13], has been found to be dispensable in *M. smegmatis* [14], although the *sigB* mutant of *M. smegmatis* is more sensitive to $H_2O_2$ and super-oxide generating compounds. The rate of transcription of the *sigB* gene increases upon exposure to high temperature, SDS, low aeration, and during stationary phase, in *M. tuberculosis* [15,16]. This suggests that *sigB* may not be essential for survival, however, it gives a competitive advantage under specific environmental conditions, like stationary growth.

In the past, several groups had identified numerous genes, which are dependent on different sigma factors by various techniques like two dimensional gel electrophoresis, promoter consensus search, in vitro transcription, and global transcriptome profiling by DNA microarray [17,18]. However, only the proteomics approach addresses the translational rate and identifies the possible postranslationally modified products. This proteomics approach is marked by three major technological advances in the past decade, namely: (i) database generated from the translated mycobacterial genome sequences, (ii) improved resolution of the highly sensitive separation technique of two dimensional gel electrophoresis, and (iii) the introduction of mass spectrometry for accurate protein identification by peptide mass fingerprinting and tandem mass spectrometry. Generation of two dimensional reference maps for mycobacterial proteins by these techniques would greatly complement their biochemical characterization and several such reports on proteomes, displaying both the cytosolic proteins and the culture filtrate proteins, exist as developed by the Berlin group and others [19–22].

Here we demonstrate the power of combining two dimensional gel electrophoresis analysis and MALDI-TOF-TOF tandem mass spectrometry based protein identification to investigate the genes that are under *sigB* regulon in *M. smegmatis*, during the stationary phase of growth. Subsequently, we also looked at the expression profile of few genes identified as targets by proteome analysis, with the help of quantitative PCR. This way we have identified a Rhamnosyltransferase which is responsible for the synthesis of polar glycopeptidolipids during stationary phase.

## Materials and methods

*Bacterial strains and culture conditions.* Wild type *M. smegmatis* $mc^2155$ [23], PMVSigB, and *sigB* mutant SM140 [24] were grown in 7H9 (Difco) medium supplemented with 2% (w/v) glucose and 0.05% (v/v) Tween 80. Recombinant clones and mutants were grown in the presence of kanamycin, added to a final concentration of 20 μg ml$^{-1}$. Cells were harvested at mid-exponential and stationary phases by centrifugation, for protein extraction.

*Chemical reagents.* All chemicals were obtained in the highest grade from Sigma unless otherwise specified. Milli-Q (Millipore) water was used for all purposes.

*Protein extraction.* Bacterial pellets were washed once with 10% (w/v) sucrose and dissolved in lysis buffer containing 50 mM NaCl, 5% (v/v) β-mercaptoethanol, 1% (v/v) IPG buffer (Amersham Biosciences), 6% (v/v) Triton X-100, and protease inhibitor cocktails (Roche) to a final concentration of 10 mg ml$^{-1}$. Cells were disrupted by sonication (Sonics Vibra Cell) for 20 min. Then the lysate was precipitated using a 2-D Clean-Up Kit (Amersham Biosciences).

*2-D PAGE.* The lysate was reconstituted with De-Streak rehydration solution (Amersham Biosciences) and then loaded on pH 3–10 and pH 4–7 linear, 18 cm Immobiline dry strips (Amersham Biosciences). IPG strips were actively rehydrated overnight with 900 μg protein and focused on an Ettan IPGphor II electrophoresis unit (Amersham Biosciences). The focused strips were equilibrated in SDS buffer (6 M urea, 2% (w/v) SDS, 50 mM Tris, pH 8.8, 30% (v/v) glycerol, and 0.002% (w/v) bromophenol blue) with 64 mM DDT and 135 mM iodoacetamide for reduction and alkylation successively. Second dimension was run on vertical 10% and 15% acrylamide SDS–PAGE gels, prepared as mentioned in Amersham Biosciences handbook [25], in a Hoeffer SE-600 unit (Amersham Pharmacia). Gels were stained by silver and wet gels were scanned in an Image Scanner II (Amersham Biosciences). For preparative runs, 2 mg of proteins was loaded in each IPG strip and gels were stained with Coomassie brilliant blue.

*Data analysis.* The digitized gel images were imported onto Ettan Progenesis software (Amersham Biosciences), protein spots were detected in each gel against the background, and then spots in each gel were matched by overlaying and warping to the reference gel. Analysis included spots that are unique in one set of gels or differ in intensity from the reference gel by a factor of 3.

*Protein identification by MALDI-TOF-MS–MS.* Protein spots of interest from Coomassie stained preparative gels were excised into $1 \times 1$ mm pieces and then destained in 50 mM $NH_4HCO_3$ in 50% (v/v) acetonitrile, twice for 15 min. The destained gel pieces were dehydrated in acetonitrile for 5 min, dried by speed vac (Savant), and reswelled in 25 μl of 50 mM $NH_4HCO_3$ containing 0.02 μg μl$^{-1}$ sequencing grade modified trypsin (Promega). The reswelled gel pieces were incubated at 37 °C for overnight. After centrifugation, the supernatant was transferred to a sterile micro-centrifuge tube, the tryptic peptides were extracted twice with 0.1% (v/v) TFA in 60% (v/v) acetonitrile and added to the supernatant. Peptides were concentrated by speed vac and dissolved in 0.1% (v/v) TFA in 50% (v/v) acetonitrile. Peptides were mixed with an equal volume of matrix solution (α-cyano-4-hydroxy cinnamic acid), spotted for analysis by MALDI-TOF-TOF (ULTRA FLEX TOF-TOF, Bruker Daltonics) instrument equipped with a pulsed $N_2$ laser, and analyzed in the reflectron mode using, a time delay of 90 ns, accelerating voltage of 25 kV in the positive ion mode. Initially spectra of 200 laser shots were acquired and the spectra were calibrated externally to a spectrum of mixture of peptides of known masses ranging from 1046 to 2465 Da. The most intense peaks in the spectrum were selected for fragmentation by laser induced dissociation (LID) using the LIFT program of ULTRA FLEX TOF-TOF instrument. For tandem mass spectrometry an average of 1000 laser shots were accumulated and the spectrum was calibrated internally to the precursor ion mass. These MS–MS spectra were used for sequence specific search in the Mascot database (Matrix Science, UK). In addition, PMF based searches were done using only the set of peptide masses, in the same database without any constraints for p*I* and molecular mass, and with a cut-off of 30% for sequence coverage. The whole procedure, right from the step of gel excision, was repeated several times to ensure correct protein identification.

*RNA extraction.* Bacteria from 5 ml cultures were pelleted, beaten with 0.5 mm zirconia/silica beads (Biospec products), and thereafter RNA extraction was performed using Auprep RNA Mini kit. The eluted total RNA was cleaned for chromosomal DNA contamination with a DNaseI (MBI Fermentas) treatment and used for the cDNA synthesis. Three independent extractions were done for each experiment reported.

*Real time PCR with Sybergreen.* The extracted total RNA was used for synthesizing first-strand cDNA with random hexamers from the Brilliant QRT-PCR core reagent kit, 2-step (Stratagene), as per the manufacturer's instruction. The cDNA thus obtained was amplified using specific sense and antisense primers (sequences in Table 3). 1× SyberGreen (Sigma) was used for, real time monitoring of amplification which was then normalized to a passive reference dye, ROX. The PCR conditions were identical for all

reactions, briefly an initial activation of Uracil-DNA-*N*-glycosylase was carried out at 50 °C for 2 min followed by initial denaturation at 95 °C. Thereafter, 39 cycles, comprising of 95 °C for 15 s, 65 °C for 1 min, and 72 °C for 30 s, were repeated and a final extension of 72 °C for 2 min was given before analyzing the nonspecific products by obtaining a melting curve. Triplicate reactions were performed together without template and without Reverse Transcriptase as negative control in each experiment. Quantitative analysis of the data obtained was done on the same OpticonMONITOR, thermal cycler (MJ research) using the data analysis software. Relative quantitation of the amount of mRNA present was estimated by comparing to the amounts of genomic DNA with the help of a standard curve. The results hence obtained were normalized to the amount of *rpoC* (mycobacterial RNA polymerase β′ subunit) that was found to be constant in all the conditions tested.

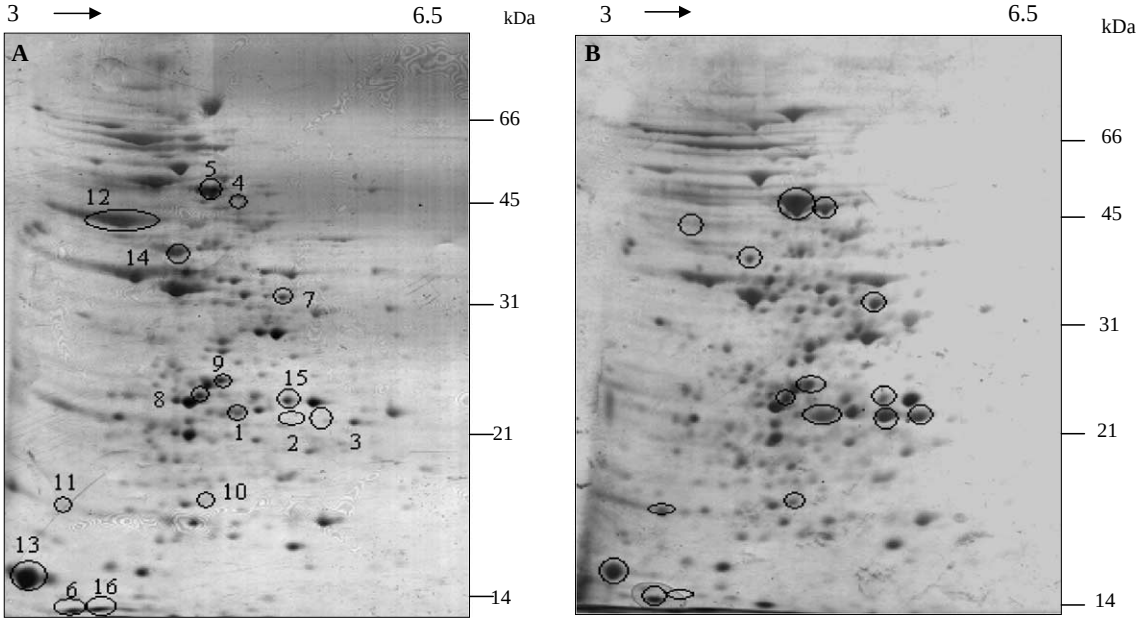


Fig. 1. 2-D protein profile of *M. smegmatis* wild type mc$^2$155 (A) and PMVSigB (B) during exponential growth. Spot numbers correspond to Table 1. Second dimension was run on a 10% SDS–PAGE gel.

Table 1
Summary of all the proteins characterized in this investigation

| Spot No. | Protein | Function | Sequence/sequence coverage for the PMF results |
|---|---|---|---|
| *Exponential phase proteins* | | | |
| Unique | | | |
| 2 | RtfA (*M. avium*) | Rhamnosyltransferase | **RELGLPTATVASPRR** |
| 3 | SucD (*M. avium*) | Succinyl-coenzymeA synthetase | **VIVMIGEIGGDAEER** |
| More abundant | | | |
| 4 | KCS | Ketoacyl coenzymeA synthetase | **SFGGPQNTNDGVTIAR** **AAVEEGIVTGGGAALVQAR** **AFLDDLAIVTGGQVVNPDVGLLLR** |
| 5 | GroEL (*M. leprae*) | Heat shock protein | **GYISGYFVTDAER** |
| 6 | GroES (Mycobacteria species) | Heat shock protein | 51.1% |
| 7 | AtoB | Acetyl-coenzymeA acetyltransferase | **MCLSGLDAIALADQLIR** |
| Less abundant | | | |
| 12 | GlnA | Glutamine synthetase | **DGQPLFHDESGYAGLSDIAR** |
| 13 | MspA | Porin precursor | **EWFHSGR TLTVQQWDTFLNGVFPLDR** |
| 14 | Fmd | Formamidase | **YLDSHLSYQR** |
| *Stationary phase proteins* | | | |
| 1 | Arr-1 | Rifampin ADP-ribosyl transferase | 46.9% |
| 2 | Ssb | Single strand binding protein | **TVVEVEVDEIGPSLR FTPSGAAVANFTVASTPR** **AGDTTITVVGNLTADPELR** |
| 3 | DNA binding protein (*C. efficiens*) | DNA binding protein | 39.2% |

Sequences obtained from MALDI-TOF-TOF spectra are in bold letters. Organisms to which *M. smegmatis* proteins have homology in the MASCOT database are indicated in parentheses.

## Results

### 2-D PAGE analysis

In order to identify the proteins which are regulated by *sigB*, we have compared the total cellular protein profile of mc$^2$155 strain with that of PMVSigB. It should be mentioned here that the SigB used in this study was from *M. tuberculosis*, which has 93% identity with *M. smegmatis*. 2-D gel analysis was carried out within a pH range of 3–10 and we observed that most of the cellular proteins were focused in the acidic pH range. Thus in subsequent experiments, a pH range of 4–7 was employed. Some evidence suggests that SigB is a sigma factor, which functions in the stationary phase. We have observed that the colony morphology of PMVSigB appears to be smooth and round as reported for a carbon starved culture of mc$^2$155 [24,26]. Thus, it was our interest to compare the proteome of these two cultures at their exponential phase.

Analysis of four independent experiments revealed three unique spots (spots 1, 2, and 3), eight spots that were more abundant (spots 4, 5, 6, 7, 8, 9, 10, and 11), and five spots (spots 12, 13, 14, 15, and 16) that were lesser in intensity by a factor of 3 in the *sigB* over-expressing condition compared to wild type, at the exponential phase (Fig. 1). Table 1 shows 9 of the 16 spots identified this way by tandem MS–MS analysis. Remaining spots were difficult to identify as they have less amount of proteins. This could also be due to the fact that either the proteins do not have homologues in the mycobacterial database or have homologies to proteins of hypothetical functions.

Later, the protein profile of the mc$^2$155 *sigB* mutant SM140 was compared to those of wild type cells at the late stationary phase. This acts as a corollary of the previous set of experiments. 2-D gels for mc$^2$155 wild type and *sigB* mutant were run in two different percentages of acrylamide (10% and 15%) in order to capture all the proteins of different molecular weights. However, only the higher percentage of acrylamide gel where low molecular weights proteins are resolved better is shown here (Fig. 2). In the *sigB* knockout condition, nine proteins were found to be less abundant than in the corresponding wild type 2-D gel profile. Out of which seven spots were resolved in 15% acrylamide gel (Fig. 2), whereas two other proteins were detectable in the 10% acrylamide gel (data not shown). Upon comparing with the identified proteins from PMVSigB, we observed that these two proteins are common with the spots 2 and 3 of Fig. 1 (PMVSigB at exponential phase). This way we tried to identify the genes that are regulated by SigB, in *M. smegmatis*. Subsequently, all these spots were also matched with the 2-D protein profile of *M. tuberculosis* at the MPIIB, Berlin database ([27], www.mpiibberlin.mpg.de/2D-PAGE/). Table 2 shows the identity of the spots obtained in the present case with *M. tuberculosis* H37RV cellular proteome database. Positions of few proteins spots from exponential protein profile (spots 3, 5, and 7) and spot 2 of the stationary phase profile

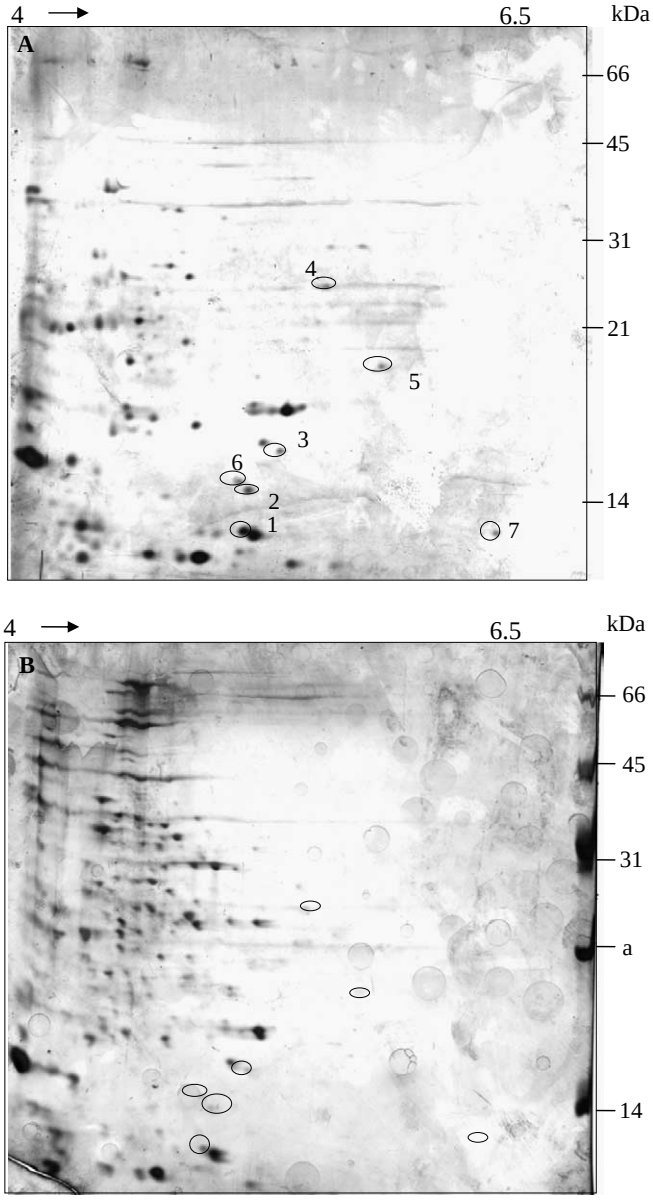


Fig. 2. 2-D protein profile of *M. smegmatis* wild type mc$^2$155 (A) and *sigB* null mutant (B) during stationary phase. Spot numbers correspond to Table 1. Second dimension was run on a 15% SDS–PAGE gel.

matched. However, other protein spots could not be traced in the *M. tuberculosis* cellular proteome map. This may be due to lack of protein homology between the two organisms or due to the different conditions for 2-D electrophoresis employed in the two studies, that is IPG strips instead of carrier ampholytes used by the Berlin group. Our main concern was to detect the proteins that were over-produced in the PMVSigB at the exponential phase, in one hand, and the proteins whose genes were downregulated in *sigB* null mutant at the stationary phase, on the other hand. For protein identification, we directly looked for sequence specific MS–MS based database search whenever we were able to observe successful fragmentation of the most abundant peptide fragment in a MALDI-TOF-TOF mass spectrom-

Table 2
Positions of the mycobacterial cellular proteins studied here, in the MPIIB Berlin 2-D database [27]

| | Proteins in this investigation | Position in *M. tuberculosis* H37RV 2-D map |
|---|---|---|
| Exponential phase | Rhamnosyltransferase | NA |
| | Succinyl-coenzymeA synthetase | + |
| | Ketoacyl coenzymeA synthetase | + |
| | GroEL | + |
| | GroES | − |
| | Acetyl-coenzymeA acetyltransferase | − |
| | Glutamine synthetase | NA |
| | Porin precursor | Unique to *M. smegmatis* |
| | Formamidase | NA |
| Stationary phase | Rifampin ADP-ribosyl transferase | Unique to non-tuberculous Mycobacteria |
| | Single strand binding protein | + |
| | DNA binding protein | NA |

+, indicates identical location; −, indicates non identical location; NA, indicates position not defined.

eter. A representative MS–MS spectrum is shown in Fig. 3. However, we also searched for peptide mass fingerprint (PMF) when the fragment spectra were not prone to further analysis. It should be mentioned here that we preferred sequence based MS–MS search for the following reasons.

Table 3
List of oligonucleotides used in this study

| Gene | Sense primer (5′ → 3′) | Antisense primer (5′ → 3′) |
|---|---|---|
| *gtf1* | ATGTTCCACTCGTCACGCTG | ACCAATCGAACGCGGTCATC |
| *gtf2* | TCACGAGGTTTGCGTTGCAG | GCATCCAGAAATTGCGAAGGAAA |
| *gtf3* | GACGATCTCTCAGTGGGAGGA | CAGGCTGCTCGAAACCTACC |
| *rpoC* | CCCGAAGGGTGAGTTCATCC | GTGTTGATGAAGTACTCCAGCAC |

Often low scoring hits appear when the search is based solely on peptide mass, observed molecular mass and observed p*I*. This is due to the presence of post-translationally modified proteins and therefore cannot be matched with the translated genome database Table 3).

### *Gene expression analysis*

We have recently reported that upon over-expressing *sigB*, the cell surface glycopeptidolipids (GPLs) of *M. smegmatis* get hyperglycosylated, similar to what is observed during carbon starvation [24]. GPLs are found among the lipids of the outer layer of some non-tuberculous fast-growing mycobacteria like *M. avium*, *M. chelonae*, etc., and in the saprophyte *M. smegmatis*. However, they are replaced by phenolicglycolipids (PGLs) in the virulent *M. tuberculosis* [28,29].The GPL locus of *M. smegmatis* houses three putative gylcosyltransferases, namely *gtf1*, *gtf2*, and *gtf3* [30]. Therefore, one of these three gylcosyltransferases (Gtf) is expected to have a def-

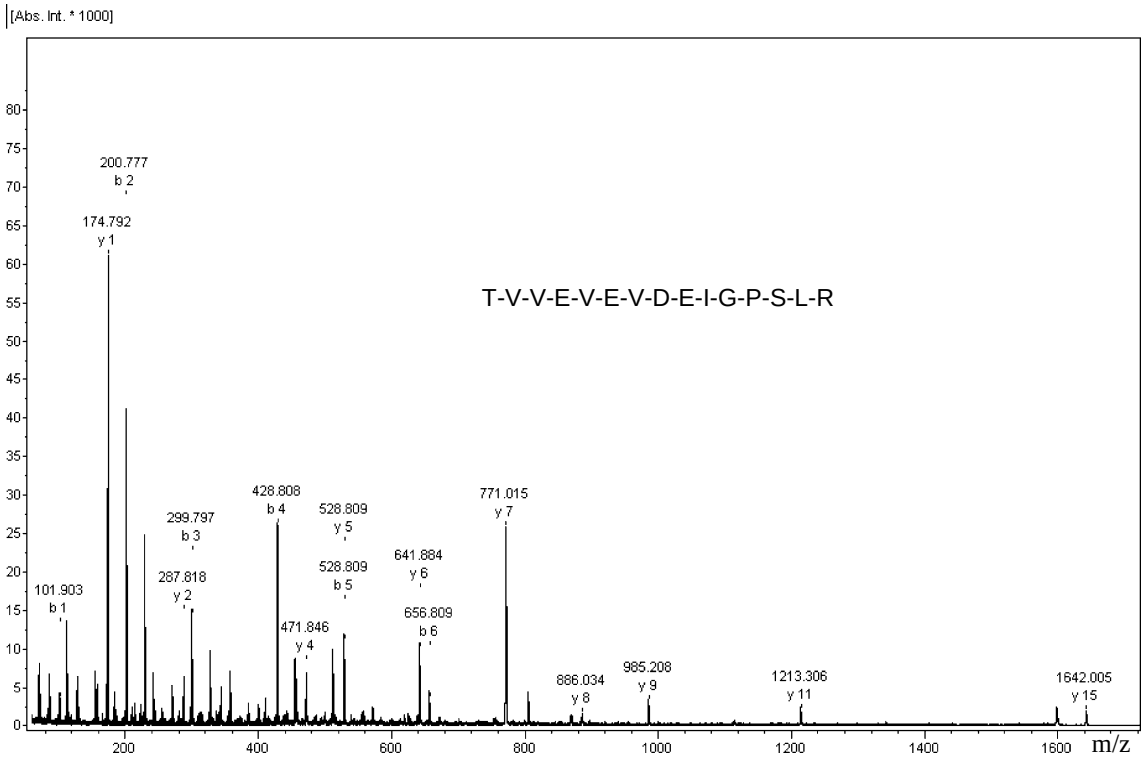


Fig. 3. Representative LID MS–MS spectra of the peptide fragment for *M. smegmatis* Ssb protein, of precursor mass 1642.005 Da. (The sequence of the peptide is shown as an inset.)

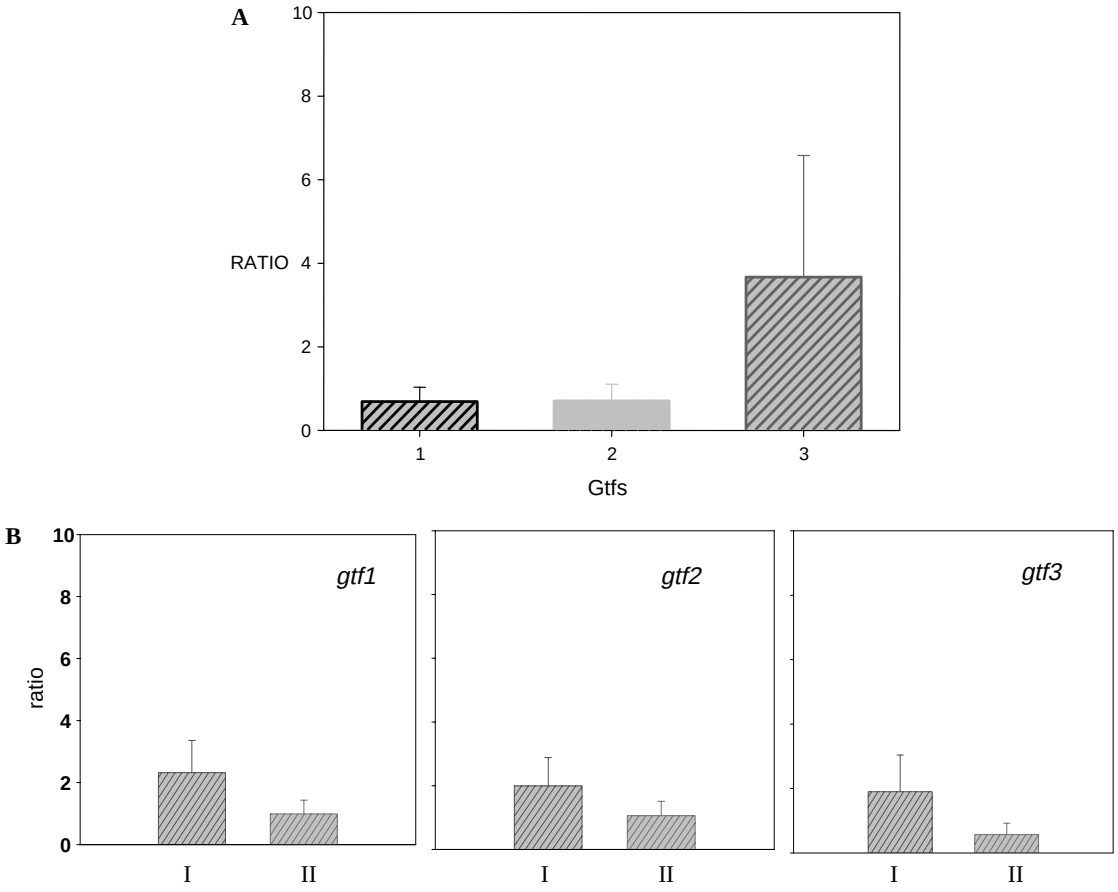


Fig. 4. (A) Comparative mRNA levels of the three glycosyltransferases (Gtf) in mc$^2$155 at the stationary phase to their exponential phase of growth. The values are expressed as ratio of the average number of cDNA copies detected in stationary phase and exponential phase. (B) Comparison of the mRNA levels of the Gtfs in PMVSigB with mc$^2$155 at the exponential phase of growth (I) and SM140 with mc$^2$155 at the stationary phase of growth (II).

inite role in hyperglycosylating the apolar GPLs. It should be mentioned here that by proteomics analysis we indeed identified a Rhamnosyltransferase which is overexpressed in PMVSigB (Table 1). In order to identify the specific Gtf, their in vivo expression levels during exponential phase and stationary phase, mRNA levels in mc$^2$155 were monitored using quantitative PCR. The level of expression of *rpoC* was taken as a control and the level of expression of *gtf* genes was normalized with respect to that of *rpoC*. Thereafter the ratio of the expression of genes as a function of exponential and stationary phases was plotted. It was observed that in mc$^2$155, the expression of *gtf3* increases by about fourfold in stationary phase, while that of the others goes below par (Fig. 4A). It has been noted that upon Gtf3 overexpression, the cell surface property of *M. smegmatis* was altered and GPL composition shifted towards the triglycosylated forms [31]. In addition, upon comparing the ratio of each *gtf*s, at the exponential phase of growth in mc$^2$155, it was found that all the *gtf*s showed a marked increase in their expression levels in PMVSigB. However at the stationary phase, in SM140 the levels of all the *gtf*s were found to be similar with only a marginal decrease in the expression of *gtf3* (Fig. 4B).

## Discussion

### *sigB dependent expression of proteins*

Upon over-expression of *M. tuberculosis sigB* in *M. smegmatis*, we identified two general stress proteins like GroEL and GroES that are overproduced at the exponential phase. This may be the result of general stress due to overexpression, but the role of *sigB* to stimulate the stress condition cannot be neglected. Moreover, the up-regulation of a Rhamnosyltransferase, Succinyl-coenzymeA synthetase, β-ketoacyl coenzymeA synthetase, and acetyl-coenzymeA acetyltransferase emphasize the role for *sigB* during stationary phase. The Rhamnosyltransferase of *M. avium* to which the glycosyltransferase (spot 2) is homologous adds the first rhamnose to the 6-deoxytallose unit of the apolar GPLs to extend its oligosaccaharide appendage [32]. Unlike *M. avium*, in *M. smegmatis* these glycosyltransferases attach an extra rhamnosyl unit to the terminal L-alaninol to produce the polar hyperglycosylated GPLs which are reported to be present decorating the *M. smegmatis* cell surface at the stationary phase [26,33]. It should be mentioned here that a protein like Rhamnosyltransferase as obtained in *M. smegmatis* proteome may not

find a counterpart in *M. tuberculosis* as the enzyme in the latter case is expected to be different with a different donor and acceptor.

MspA, an *M. smegmatis* specific major porin, was found to be produced in low quantity in PMVSigB. This porin is a tetrameric pore forming complex located on the mycobacterial cell wall for trafficking hydrophilic nutrients and small molecules like sugars, amino acids, antibiotics, etc. [34,35]. Over-expression of *mspA* had been reported to promote growth and increase antibiotic susceptibility in *M. bovis* BCG and in *M. tuberculosis* [36]. Moreover, a multi-drug resistant phenotype appeared in *M. smegmatis* after deletion of the major porin *mspA* [37].

Among the metabolic enzymes, succinyl-coenzymeA synthetase, β-ketoacyl coenzymeA synthetase, and acetyl-coenzymeA acetyltransferase were found to be over-produced in PMVSigB. Succinyl-coenzymeA synthetase, the central enzyme in the TCA cycle, has been found to be up-regulated in *M. tuberculosis* bacilli isolated from infected mouse lung tissue [38]. Moreover, induction of *sucD* upon deletion of the extra-cytoplasmic sigma factor *sigE* was reported in *M. tuberculosis* [39]. β-Ketoacyl coenzymeA synthetase is one of the enzymes present in fatty acid synthase I (FAS-I), required for de novo synthesis of long chain fatty acids ($C_{16}$–$C_{26}$) in mycobacteria [40]. These long chain fatty acids serve as raw material for the synthesis of the more complex mycolic acids, which constitutes the major lipids of the thick cell wall [41]. Acetyl-coenzymeA acetyltransferase is one of the enzymes involved in fatty acid degradation, which is an alternate way for generating energy apart from glycolysis and TCA cycle, and fatty acids from dead mycobacteria may provide an alternate source of carbon during nutrient limitation [42]. Moreover, degradation of fatty acids generates two carbon compounds which form the precursors for primary metabolite biosynthesis, like polyketides [43]. FadA4, a homologue of acetyl-coenzymeA acetyltransferase, was reported to be induced in *M. tuberculosis* during infection of human macrophages [44].

All of these metabolic enzymes may have an extra function in gene regulation during stationary phase, like their eukaryotic counterparts (for review see [45]). Glutamine synthetase and formamidase were found to be under-expressed in PMVSigB. We cannot attribute any reason for their downregulation other than what has been reported for *B. subtilis* [46,47]. In the former case, it was due to stationary phase related stress and in the latter case due to over-expression of a protein.

Moreover, while comparing the stationary phase 2-D profiles of *sigB* null mutant of *M. smegmatis* with that of wild type, Rhamnosyltransferase and succinyl-coenzymeA synthetase were among the proteins which had been found to be over-produced in the wild type cells compared to *sigB* mutant. These two proteins were also over-produced in PMVSigB at the exponential phase, thus validating our argument that SigB is having a role to play during the stationary phase. Two DNA binding proteins were also identified to be less abundant in the mutant which emphasizes the need for protecting DNA against stationary phase induced damages. We have previously identified, through proteomics, a DPS (DNA binding protein from starved cells) homologue in *M. smegmatis* to be upregulated during carbon-starved condition in wild type $mc^2155$ cells [48,49]. Ssb protein, which we have detected here, may have an extra role of protecting ssDNA during recombination repair, which is high due to stationary phase. Another protein, Rifampin ADP-ribosyltransferase, which inactivates rifampicin by putting an ADP-ribosyl unit in it [50], was also found to be down-regulated in the *sigB* mutant strain.

Since we have observed hyperglycosylation of GPLs in PMVSigB at the exponential phase, we tried first to identify the particular stationary phase specific Gtf and then look for its expression in PMVSigB vis a vis SM140 during their growth phases. While our work was in progress, it was reported that, upon overexpression of Gtf3 only polar triglycosylated GPLs are produced in *M. smegmatis* [31] which infers that Gtf3 may probably be the enzyme responsible for attaching an extra rhamnose to the apolar GPLs. However, the in vivo expression levels of these *gtf*s were not addressed. From our work presented here, it can be concluded that *gtf3* expression in upregulated only at the stationary phase. On the other hand, once the apolar GPLs are synthesized, the mRNA levels of *gtf1* and *gtf2* decrease marginally. Upon blasting the protein sequences of three Gtfs to the SEARCHGTr database ([51], http://www.nii.res.in/searchgtr.html) for donor specificity, it was observed that Gtf2 has 26.1% identity to deoxyallosyl-transferase from actinobacteria, *Streptomyces fradiae*, together with conserved donor binding residues. Thus, it appears that Gtf2 is the probable candidate for transferring 6-deoxytallose to the peptidolipid aglycone.

When we looked at the expression of these *gtf*s in PMVSigB at the exponential phase, it was observed that the mRNA levels of the *gtf*s are higher than that of $mc^2155$ with a marked increase in the case of *gtf3*, which in turn is required for synthesizing extra polar GPLs in PMVSigB. While in case of a *sigB* knockout strain, the expression of either of the *gtf*s reduces considerably at the stationary phase, when compared to $mc^2155$. In addition, the stationary phase GPL profile of SM140 is largely similar to that of wild type with the presence of the polar species (data not shown). This suggests that SigB may not be the primary determining factor for *gtf*s expression in stationary phase; other proteins like different ECF sigma factors or regulators may play an important role.

In conclusion, it appears that the groups of proteins, which we have identified here by 2-D proteomics, are of different classes. One group consisting of Rhamnosyltransferase, succinyl-coenzymeA synthetase, and β-ketoacyl coenzymeA synthetase may be under the control of *sigB* in *M. smegmatis*, whereas other proteins like Ssb may have an indirect link. Succinyl-coenzymeA synthetase and single strand binding protein were among the

proteins that were found to be induced at late stationary phase in *M. smegmatis* [52]. Though it is now predicted that unlike *M. tuberculosis*, *M. smegmatis* could carry double the number of sigma factors [53], which allows us to speculate about the existence of more inter-regulatory networks between these repertoire of sigma factors. Our observations contribute to the minimum literature available on the function of SigB in *M. smegmatis*.

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