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Low expression level of *glnA1* accounts for absence of cell wall associated poly-L-glutamate/glutamine in *Mycobacterium smegmatis*

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ABSTRACT

Cell wall associated poly-L-glutamine (PLG) layer synthesis is directly linked to glutamine synthetase (GS) encoded by *glnA1* in tuberculosis causing mycobacteria. Avirulent *Mycobacterium smegmatis* (*M. smegmatis*) despite of having a *glnA1* homolog lacks cell wall associated PLG layer. In the present study, we complemented a *ΔglnA1* mutant of *Mycobacterium bovis* (lack PLG in cell wall) with *M. smegmatis glnA1* cloned under *M. bovis glnA1* promoter. PLG synthesis was restored in the cell wall of complemented strain. The complemented strain also showed increased resistance to physical stresses such as lysozyme, SDS and increased survival in THP-1 macrophages in comparison to the knockout. Further, in β-galactosidase reporter assay *M. smegmatis glnA1* promoter showed ten times less activity as compared to *M. bovis glnA1* promoter. $GACT_{-8-11} \rightarrow TGAC$ mutations in the *M. smegmatis glnA1* promoter restored its activity by 60% as compared to the activity of *glnA1* promoter of *M. bovis*. This mutation also showed increased GS expression and produced cell wall associated PLG in *M. smegmatis*. The results of this study demonstrate that *glnA1* promoter of *M. smegmatis* accounts for low expression level of GS and apparently responsible for absence of cell wall associated PLG layer.

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1. Introduction

Mycobacterium bovis (*M. bovis*), etiologic agent of bovine tuberculosis, is one of the biggest challenges for cattle farming industry. The disease continues to be one of the major reasons of premature death in humans and cattles, inflicting huge economic losses [1]. Studies involving virulent factors, in particular the promoters and other regulatory elements governing their expression can enhance our understanding of *M. bovis* pathogenesis which can in turn pave a way for development of new drugs and effective treatment measures. Moreover comparative analysis of the regulatory elements of slow growing mycobacterial species with their fast growing saprophytic counterparts may reveal interesting information about gene expression in pathogenic mycobacteria.

Nitrogen metabolism plays a crucial determining role in bacterial physiology. In nitrogen limiting conditions, glutamine synthetase (GS) and glutamate synthetase are the only means of assimilating ammonia to glutamine and glutamate in mycobacteria [2]. Very little is known about GS of *Mycobacterium smegmatis*. In contrary, GS of *M. bovis* and*M. tuberculosis* has always been associated with its virulence and pathogenicity. GS of virulent mycobacteria form a major component of secreted proteins. It was earlier reported that *Mycobacterium tuberculosis glnA1* mutant is avirulent in guinea pigs [3] and knockout of *glnA1* in *M. bovis* results in decrease in cell wall strength of the pathogen [4]. Presence of poly-L-glutamine (PLG) layer in the cell wall of virulent mycobacteria has also been attributed to its GS enzyme. Inhibiting or knocking out GS enzyme inhibits PLG synthesis in the cell wall [4–6].

M. bovis and *M. tuberculosis glnA1* exhibits 100% sequence identity (both the coding and promoter region). It has been reported earlier that in *M. tuberculosis* GS expression is 5–10 times high in nitrogen limitation as compared to nitrogen excess [7]. In nitrogen limiting conditions, expression of GS in *M. bovis* and *M. tuberculosis* is under σ60 type promoter sequence present upstream to *glnA1* gene [7,8]. GS expression levels determine PLG layer synthesis in *M. bovis*. In our previous studies we reported

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that PLG layer is absent in the cell wall of *M. bovis* when grown in nitrogen excess due to significant decrease in the GS expression levels. PLG layer formation is favored only in nitrogen limiting conditions in virulent mycobacteria due to increased GS expression [8].

It is exciting that despite of presence of a *glnA1* homolog in the genome with 84% identity to *M. bovis* counterpart, *M. smegmatis* does not express much GS protein and lack PLG layer in its cell wall and that presence of *M. bovis glnA1* locus (*glnA1* with its native promoter) in *M. smegmatis* leads to PLG synthesis in the cell wall [4]. In view of the fact, that σ60 type promoter sequence upstream to *glnA1* is responsible for increased GS expression in nitrogen limitation in *M. bovis* which in turn directs PLG formation in its cell wall; this study focused on comparative analysis of this promoter in *M. smegmatis* and *M. bovis*. The promoter region of *glnA1* in *M. bovis* and *M. smegmatis* revealed the reason for the difference in the expression levels of GS in the two species and ultimately lack of PLG layer in the cell wall of *M. smegmatis*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The plasmids and bacterial strains used in this study are listed in Table S1. 7H9 broth (Difco) supplemented with 10% (v/v) albumin, dextrose and catalase (ADC), 0.2% (v/v) glycerol and 0.05% (v/v) Tween 80 was used for growth of *mycobacterial* strains routinely. The cultures were grown at 37 °C with shaking at 150 rpm. *Escherichia coli* DH5α was grown in Luria–Bertani medium. Kanamycin was added at concentration of 25 μg/ml for mycobacteria and 50 μg/ml for *E. coli* strains. Experiments involving virulent strain were performed in Bio-safety Level-3 laboratory at Jawaharlal Nehru University.

2.2. Cloning of *M. smegmatis glnA1* gene with its native and with *M. bovis glnA1* promoter and complementation of *ΔglnA1* mutant of *M. bovis*

Cloning was performed using standard procedures. The *M. smegmatis glnA1* along with its 320 bp upstream region was amplified using *M. smegmatis* genomic DNA as a template with primers 1/2 (Table S2). The amplified DNA was ligated in pMV261, *E. coli–Mycobacterium* shuttle vector, producing pSD1 and electroporated into *M. smegmatis* mc2 155. The transformed strain was designated SD1.

Further, *M. smegmatis glnA1* gene was fused with *M. bovis glnA1* promoter by megaprimer PCR. Primer 3 was designed from 3′ end of *M. bovis glnA1* promoter containing 5′ flanking end of *M. smegmatis glnA1* gene. PCR amplification was carried out to amplify *M. bovis glnA1* promoter and *M. smegmatis glnA1* gene by using primers 3/5 and primers 2/4 respectively (Table S2). Gene fusion was done by PCR amplification by using PCR product of the above. Next, fused gene was amplified by using primer 5/2. The amplified DNA fragment was ligated in pMV261, producing pSD2 plasmid. The resulting construct pSD2 was electroporated into wild type *M. smegmatis* strain and the transformed strain was designated SD2.

For complementation study, *M. smegmatis glnA1* gene with *M. bovis glnA1* promoter was amplified by using pSD2 as template with primers 5/2, and ligated in pNBV1 vector, producing pSD3 plasmid. The resulting construct pSD3 was electroporated in *ΔglnA1 M. bovis* strain (6) and the transformed strain was selected on 7H10 plates containing hygromycin and designated as SD3.

2.3. Determination of GS expression and activity in mycobacterial strains

Strains were grown till exponential phase and cell pellets were harvested. The total cell lysate was prepared as described previously [8]. The intracellular GS activity was measured by γ-glutamyl transfer reaction as described previously [8,9]. The intracellular protein expression was determined by SDS-PAGE and western blotting by anti-GS antibody.

2.4. Isolation and estimation of PLG in mycobacterial strain

Cell wall was prepared from the cell pellet of exponential phase cultures grown in 7H9 medium. The PLG was purified and confirmed by GC–MS analysis as described earlier [8,10].

2.5. Intracellular growth of mycobacterial strains in THP-1 macrophages

Intracellular growth of *M. bovis* strains (WT, *ΔglnA1* and complemented) was performed as described earlier [4].

2.6. SDS and lysozyme sensitivity test

M. bovis strains were grown till exponential phase (15 days; OD_{600} ranging from 1.2 to 1.4), and 6 μl of 10-fold diluted culture was spotted onto 7H10 agar plates containing 10% OADC, 0.05% Tween 80 and 0.01% SDS. Lysozyme sensitivity assay was performed as described earlier [4].

2.7. Immungold localization of PLG by transmission electron microscopy

Immunogold localization of *M. bovis* strains (WT, *ΔglnA1* and complemented) was done as described earlier [8].

2.8. Construction of *M. bovis* $glnA1_{pro}$-*lacZ* and *M. smegmatis* $glnA1_{pro}$-*lacZ* transcriptional fusions and site-directed mutagenesis of *M. smegmatis glnA1* promoter region

M. bovis and *M. smegmatis glnA1* promoter (224 bp upstream of the start codon) were amplified by PCR using primers 6/7 and 8/9. Both PCR products were ligated into pSK8 *lacZ* reporter vector [13]. These generated constructs were digested by KpnI/HindIII and ligated into pMV261. The generated $glnA1_{pro}$-lacZ fusion construct pSD4 and pSD5 were then electroporated in *M. smegmatis* for assessment of promoter activity by β-galactosidase assay. The transformed strains thus obtained were designated as SD4 and SD5 respectively.

Oligonucleotide mutagenesis was done using Quik Change Site-directed Mutagenesis Kit (Stratagene) according to the instruction of the manufacturer. Plasmid pSD5 (carrying the wild-type *M. smegmatis glnA1* promoter-*lacZ* gene fusion) and pSD1 (carrying the wild-type *M. smegmatis glnA1* gene with promoter) were used as template for mutations with primers listed in (Table S2). The resulting plasmids contained the following mutations in the *M. smegmatis glnA1* promoter in pSD5: ΔCA_{49} (pSD6), $G_{-30} \rightarrow T$ (pSD7), $TC_{-20\text{-}21} \rightarrow CG$ (pSD8) and $GACT_{-8\text{-}11} \rightarrow TGAC$ (pSD9). These plasmids were used to transform wild type *M. smegmatis* strains. The mutation $GACT_{-8\text{-}11} \rightarrow TGAC$ was also created in pSD1 producing pSD10, which was then transformed in wild type *M. smegmatis* (Table S1). The resulting strain SD10 was then further screened for GS expression and PLG isolation experiments.

2.9. Assessment of promoter activity by β-galactosidase assay

β-galactosidase assay was performed as described earlier [11]. The cultures were grown till log phase and about 2 ml culture was harvested at various time points. Average of triplicate samples, was determined. The experiment was performed for three times and average of the three experiments was plotted.

3. Results

3.1. GS expression and activity in mycobacterial strain

Exponential phase culture of wild type *M. bovis*, *ΔglnA1*, wild type *M. smegmatis*, SD1, SD2 and SD3 strains were harvested and intracellular GS expression and activity was determined. It was observed that in WT *M. smegmatis* and in SD1 (*M. smegmatis glnA1* gene with native promoter) the GS expression was not detectable while GS expression in SD2 (*M. smegmatis glnA1* gene with *M. bovis glnA1* promoter) was observed at ~58 kDa. GS expression was observed at ~58 kDa in both *M. bovis* and in SD3 while the same was absent in *ΔglnA1* (Fig. 1A). Similar was the case with intracellular GS activity in all the strains. The GS activity in wild type *M. smegmatis* and SD1 (used as control) was around 0.4 U/mg while in SD2 the GS activity was observed to be 2.4 U/mg (Fig. 1B). In SD2, presence of *M. bovis glnA1* promoter upstream to *glnA1* of *M. smegmatis* resulted in increased transcription and in turn increased GS expression and activity. In contrast to the knockout GS activity in the complemented SD3 strain was high and almost similar to activity in wild type *M. bovis* (Fig.1C).

3.2. Estimation of PLG in mycobacterial strain

In contrary to the wild type *M. smegmatis* and SD1 strains where there was no detectable PLG found, PLG was purified from the cell wall (Table S3) and it was analyzed by GC MS analysis (data not shown). Cell wall associated PLG was restored in the SD3 complemented strain in contrast to the knockout which lack PLG. Presence of PLG layer in the cell wall of SD2 can be attributed to high GS expression and activity in contrast to wild type *M. smegmatis* and SD1. It has been reported earlier that GS of *M. tuberculosis* and *M. bovis* play a direct role in PLG layer synthesis in the cell wall of the virulent mycobacteria [4,5]. From our results we concluded that GS of *M. smegmatis* can equally contribute in PLG layer synthesis in the cell wall. This further validated that GS of *M. smegmatis* can also direct PLG synthesis if its expression level is comparable to the GS expression levels in virulent mycobacteria.

3.3. Effect of GS expression on intracellular growth and cell wall sensitivity of mycobacteria to SDS and lysozyme

The complemented strain SD3 showed increased survival compare to Δ*glnA1* mutant in THP-1 cells (Fig. 2A). This may be due to the altered cell wall chemistry or presence of PLG layer in the cell wall. This was in accordance with earlier findings that *glnA1* is important for intracellular growth of *M. bovis* and *M. tuberculosis* [3,4].

Harish et al. 2010 reported that *glnA1* knockout of *M. bovis* is more susceptible to physical and chemical stress [4]. In the present study, we observed that the defect in SDS tolerance of the *M. bovis* Δ*glnA1* mutant was restored by complemented strain SD3 (Fig. 2B). Likewise, the complemented strain SD3 showed two fold increase in resistance to lysozyme treatment in comparison with the Δ*glnA1* strain (Fig. 2C). The result was in accordance with the fact that apparently, loss of PLG affects the strength of *M. bovis* cell wall [4].

3.4. Immunogold localization of PLG in the cell wall by transmission electron microscopy

In order to validate the above results i.e. complementation of Δ*glnA1* mutant of *M. bovis* restores PLG formation in the cell wall, immunogold localization was performed. Gold particles were observed at the cell periphery of the wild type *M. bovis* and SD3 which confirmed the presence of PLG layer in the cell wall of these strains (Fig. S1). However, no gold particles were seen in the cell periphery of the Δ*glnA1* mutant which can be attributed to the lack of PLG layer (Fig. S1). This result was in accordance with the previous findings that PLG synthesis in the cell wall is directly linked to the presence of *glnA1* gene [4].

3.5. Assessment of promoter activity by β-galactosidase assay in *M. bovis* $glnA1_{pro}$-*lacZ* and *M. smegmatis* $glnA1_{pro}$-*lacZ* transcriptional fusions and effect of mutations in glnA1 promoter

Strains were grown till log phase, cells were harvested and β-galactosidase assay was performed as described earlier. The β-galactosidase activity in *M. smegmatis* $glnA1_{pro}$-*lacZ* transcriptional fusion was approximately ten times less than that in *M. bovis* $glnA1_{pro}$-*lacZ*. No activity was found in *M. smegmatis* as vector control (pMV261 with only *lacZ* gene) (Fig 3A). This result suggests that GS in *M. bovis* has higher expression as compared to *M. smegmatis* GS. It is in agreement with earlier reports which stated that GS expression in virulent mycobacteria is very high while in *M. smegmatis* is below detectable limits [12–14].

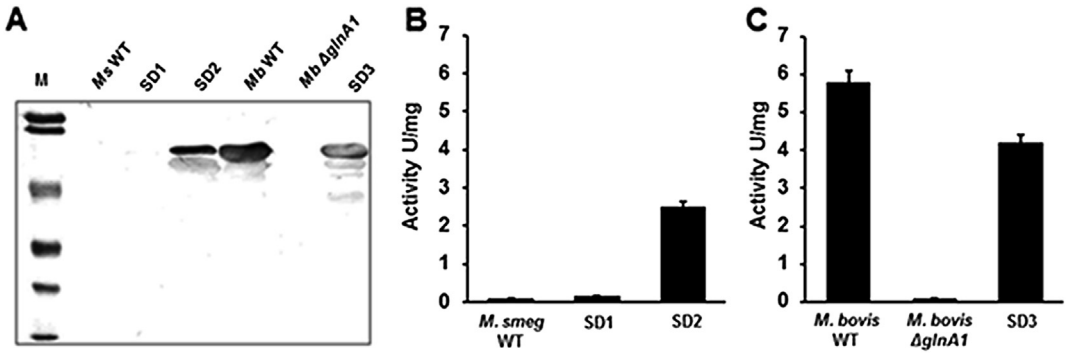


Fig. 1. GS expression and enzyme activity of mycobacterium strains. A. Western blots of the intracellular fractions analyzed by the anti-GS antibodies. B. Enzyme activity in the intracellular fractions. Lane M, marker; *Ms* WT, *M. smegmatis* wild type; *Mb* WT, *M. bovis* wild type; *Mb ΔglnA1*, *M. bovis ΔglnA1*. Data are mean ± SEM of triplicate tubes and are representative of three independent experiments.

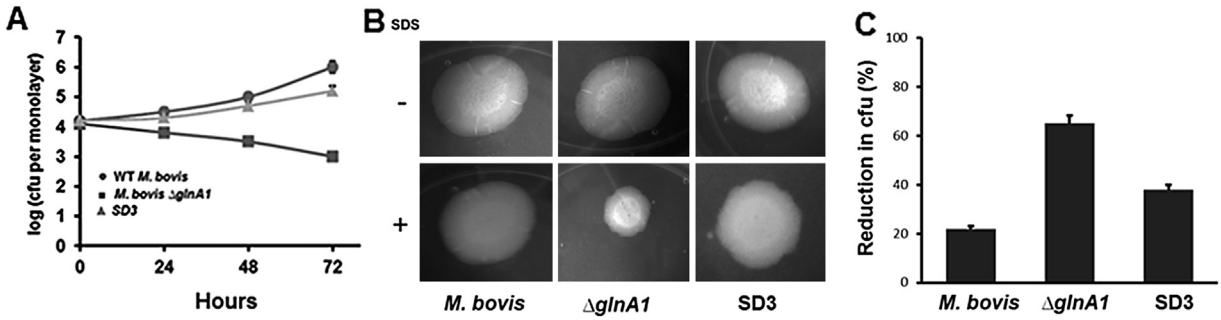


Fig. 2. A. Intracellular growth of M. bovis, mutant and complemented strains (SD3) in THP-1 cells. Cells were cultured in RPMI medium containing 2 mM L-glutamine. (●) Wild type *M. bovis*; (■) *ΔglnA1* mutant; (▲) SD3. The data presented are mean ± SEM of triplicate wells and are representative of three individual experiments. B. Sensitivity of mutant to SDS. Late log phase cultures of the three strains were spotted on SDS and normal 7H10 plates and grown for 15 days. C. Sensitivity of mutant to lysozyme. After lysozyme treatment cultures were serially diluted and plated on 7H10 plates and observed. The experiment was repeated three times. Data are mean ± SEM of three independent experiments.

Unidentical bases of *M. smegmatis glnA1* promoter were substituted with the bases that are present in *M. bovis* by site directed mutagenesis (Fig. 3C). β-galactosidase activity was performed to evaluate the gain in promoter function after mutations (Fig. 3B). It was seen that there was 60% significant increase in the promoter activity in SD9 (mutant containing GACT$_{-8\text{-}11}$ → TGAC). The gain in promoter function indicated that the −8 to −11 region is important for promoter activity. SD6 and SD7 showed no significant

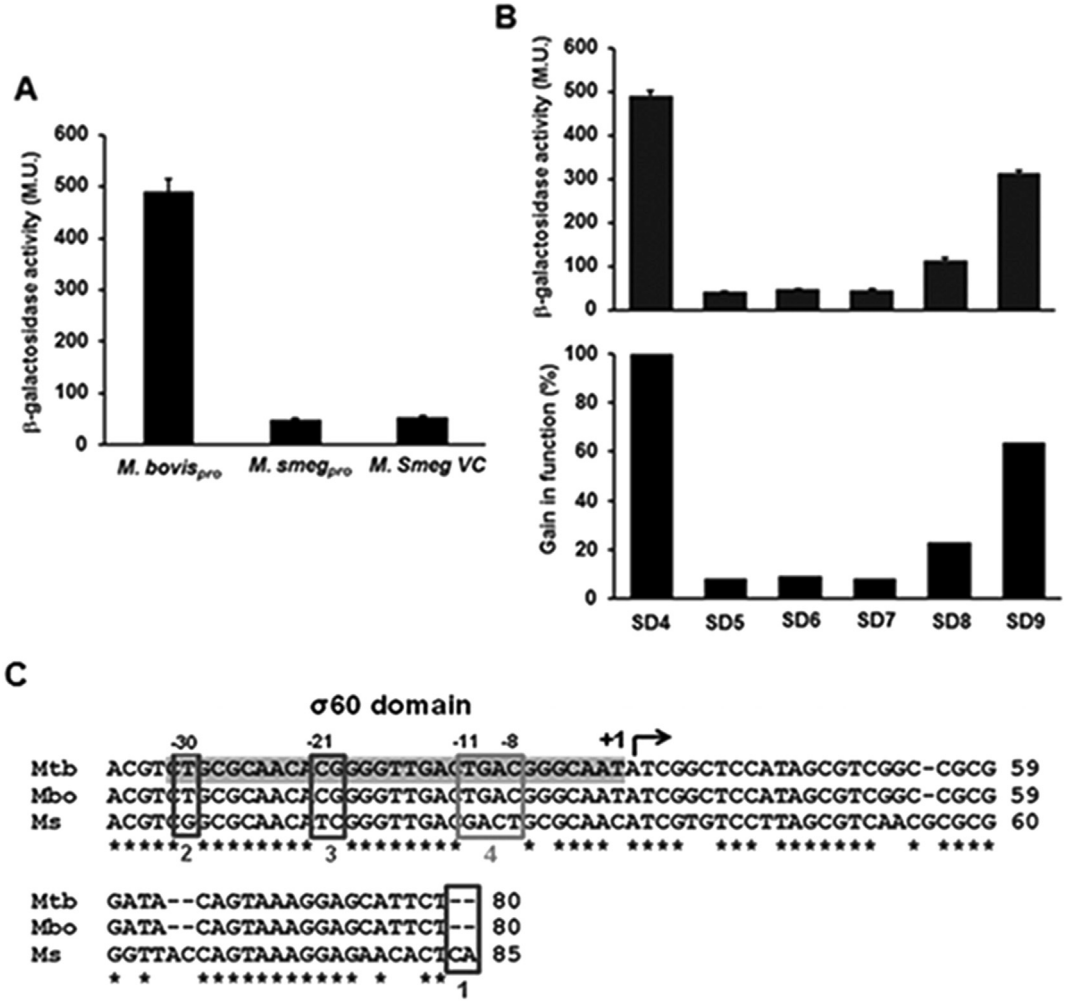


Fig. 3. Promoter activity by transcription fusion of *glnA1* promoter with *lacZ* gene. A. *M. bovis*/*M. smegmatis glnA1*$_{pro}$-*lacZ* transcriptional fusion variants in *M. smegmatis* were grown in 7H9 medium. Cells were harvested and β-galactosidase activity was determined. B. Site directed mutagenesis was done in the *M. smegmatis glnA1* promoter and β-galactosidase activity was determined to study the effect of mutation in promoter activity. Gain in function (lower panel) was calculated by taking β -galactosidase activity of SD4 as 100%. Values are the average of three independent experiments, each assayed in duplicate. Error bars represent the SD. M.U. − Miller Units. C. Scheme for site directed mutagenesis in σ60 domain of *glnA1* promoter. The unidentical bases in *glnA1* promoter of *M. smegmatis* were substituted with those present in *glnA1* promoter of *M. bovis* by site directed mutagenesis. Box represents the replaced bases by site directed mutagenesis. 1, CA bases was deleted; 2, G$_{-30}$ → T; 3, TC$_{-20\text{-}21}$ → CG; 4, GACT$_{-8\text{-}11}$ → TGAC. Gray box, σ60 domain; Mtb, *M. tuberculosis*; Mbo, *M. bovis*, Ms, *M. smegmatis*. The black arrow and +1 represents the transcriptional start site. The number on the top of the base represents the position from the transcriptional start site; * represents the identical bases.

increase in the promoter activity, although there was a little increase in the activity in SD8 (TC_{-20-21} → CG). *glnA1* promoter of *M. tuberculosis* and *M. bovis* contains a σ60 type promoter [7,12] (σ60 is popularly known as sigma54 and sigma N). σ60 type promoters are known to play a role in nitrogen metabolism. It has been reported earlier that −12 and −24 regions of σ54 promoters are crucial for promoter activity [15,16].

3.6. Effect of mutation in the M. smegmatis glnA1 promoter on GS expression and PLG synthesis

To validate the above results the mutation $GACT_{-8-11}$ → TGAC was created in pSD1 (*M. smegmatis glnA1* with native promoter) producing pSD10. The mutation $GACT_{-8-11}$ → TGAC in the promoter increased GS expression (Fig 4A) in the SD10 strain which resulted in PLG synthesis in the cell wall (Fig 4B). This result suggests that the expression of GS enzyme directs synthesis of PLG in the cell wall of mycobacteria.

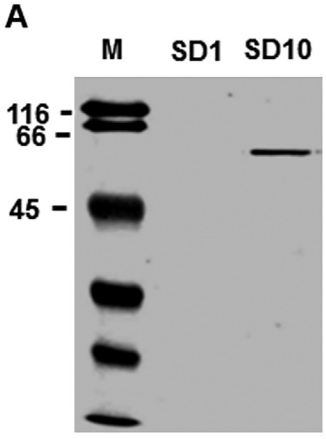
4. Discussion

The unique chemistry of mycobacterial cell wall accounts for its inherent resistance to common antibiotics and adverse conditions in the host cell [17,18]. Virulent strains of mycobacteria possess poly-L-glutamate/PLG in the cell wall [19–21]. GS of virulent mycobacteria is implicated in providing glutamine for the synthesis of PLG layer in the cell wall, which accounts for about 10% of the cell wall weight. There are direct evidences for the involvement of *glnA1* (coding for GS) in the synthesis of PLG layer and its role in invigorating the cell wall of the pathogenic species [4,5]. In view of the fact that saprophytic *M. smegmatis* has *glnA1* homolog in the genome with 84% identity to the *glnA1* of virulent species (*M. bovis*), it is intriguing that PLG is absent in cell wall of *M. smegmatis*.

Our previous studies showed that high nitrogen conditions inhibit PLG synthesis in the cell wall of *M. bovis*, because of significant decrease in the expression level of the GS enzyme [8]. This incited us to investigate that absence of PLG layer in the cell wall of *M. smegmatis* is because of significantly low expression levels of GS. The results described provide evidence for a role of high expression level of GS, governed by *glnA1* promoter in synthesis of PLG layer in the cell wall of *M. bovis*. Expression of *M. smegmatis glnA1* under *M. bovis glnA1* promoter (SD2 strain) leads to increased GS expression and synthesis of PLG in *M. smegmatis*. This data indicated that a low expression level of GS in *M. smegmatis* is responsible for absence of PLG layer in the cell wall. This was further validated by complementation of *ΔglnA1* of *M. bovis* (which lack PLG layer) with *M. smegmatis glnA1* expressed under *M. bovis glnA1* promoter (SD3 strain). PLG synthesis and GS expression was restored in the complemented strain almost comparable to the wild type *M. bovis*. The ability to survive in THP macrophages and cell wall defect was also restored in the complemented strain as compared to the knockout strain. This could be credited to the restoration of PLG layer in the complemented strain. Our previous findings showed that PLG layer is important for cell wall strength in *M. bovis* [4]. The complemented strain exhibited increased resistance to SDS and lysozyme treatment as compared to the *ΔglnA1* strain.

GS in virulent mycobacteria is secreted in large amounts in the extracellular medium and is one of the profusely expressed protein in virulent mycobacteria [7,13]. A high expression level of GS enzyme in virulent mycobacteria is attributed to *glnA1* promoter (σ60 promoter) upstream to the coding sequence [12,13]. Since GS expression level is very low (below detectable limits) in *M. smegmatis*, it was speculated that it can be due to difference in σ60 promoter of the virulent and the avirulent species. In the light of the above facts, we thought to investigate the promoter activity of *M. smegmatis glnA1* and *M. bovis glnA1* by reporter system. β-galactosidase assay results showed a significant ten times difference in the promoter activity of *M. bovis glnA1* promoter and *M. smegmatis glnA1* promoter. Alignment of *M. smegmatis* and *M. bovis glnA1* promoters (σ60 promoter) respectively showed 81% identity. Further, critical bases responsible for the difference in the promoter activity were studied. The unidentical bases or regions in *M. smegmatis* promoter were mutated with those that are present in *M. bovis*. Interestingly, $GACT_{-8-11}$ → TGAC mutation in the *M. smegmatis glnA1* promoter showed significant increase in the promoter activity as evaluated by β-galactosidase assay. Also, GS expression and PLG synthesis was restored in the *M. smegmatis* strain (SD10) containing the desired mutation. The result was in agreement with previous findings which suggest that −12 and −24 regions in σ60 type promoters have the recognition elements for binding of RNA polymerase and play an important role in promoter activity [15,16,22]. Based on our results it seems that apparently level of expression of GS enzyme plays a role in the synthesis of PLG layer in cell wall of mycobacteria.

The exact pathway of PLG synthesis in mycobacteria is not clear till date; the enzymes involved in its synthesis need to be established which can throw light on new potential drug targets against tuberculosis. In conclusion, vital role of *glnA1* promoter in PLG



Strains	Amount (mg/gm of dry weight)
Wild type	No PLG
SD1	No PLG
SD10	0.040±0.01

Fig. 4. Effect of mutation in the *glnA1* promoter on GS expression and PLG synthesis. A. Western blots of intracellular fractions analyzed by the anti-GS antibodies. B. PLG estimation in the cell wall.

synthesis through effect on GS expression is the key finding of this study. This could be useful in enhancing our understanding of gene expression in pathogenic mycobacteria and can open new possibilities for development of diagnostic and treatment measures.

Conflict of interest

No conflict of interest.

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Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.01.079.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.079.

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