Glutamine synthetase encoded by *glnA-1* is necessary for cell wall resistance and pathogenicity of *Mycobacterium bovis*

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Pathogenic strains of mycobacteria produce copious amounts of glutamine synthetase (GS) in the culture medium. The enzyme activity is linked to synthesis of poly- α -L-glutamine (PLG) in the cell walls. This study describes a *glnA-1* mutant of *Mycobacterium bovis* that produces reduced levels of GS. The mutant was able to grow in enriched 7H9 medium without glutamine supplementation. The *glnA-1* strain contained no detectable PLG in the cell walls and showed marked sensitivity to different chemical and physical stresses such as lysozyme, SDS and sonication. The sensitivity of the mutant to two antitubercular drugs, rifampicin and D-cycloserine, was also increased. The *glnA-1* strain infected THP-1 cells with reduced efficiency and was also attenuated for growth in macrophages. A *Mycobacterium smegmatis* strain containing the *M. bovis glnA-1* gene survived longer in THP-1 cells than the wild-type strain and also produced cell wall-associated PLG. The *M. bovis* mutant was not able to replicate in the organs of BALB/c mice and was cleared within 4–6 weeks of infection. Disruption of the *glnA-1* gene adversely affected biofilm formation on polystyrene surfaces. The results of this study demonstrate that the absence of *glnA-1* not only attenuates the pathogen but also affects cell surface properties by altering the cell wall chemistry of the organism via the synthesis of PLG; this may be a target for drug development.

Received 16 July 2010
Revised 17 August 2010
Accepted 17 August 2010

INTRODUCTION

Tuberculosis is a major cause of mortality and morbidity around the world. The disease is caused by different species of mycobacteria, transmitted primarily through the respiratory route into the lungs and spread to other organs through the haematogenous system (Flynn & Chan, 2001). *Mycobacterium tuberculosis* and *Mycobacterium bovis* are the two major species that infect humans and animals, inflicting huge losses in terms of human health and the economy. Therapeutic intervention is achieved through a multiple drug regimen that involves a combination of antibiotics. However, the emergence of multidrug-resistant strains has prompted worldwide efforts to develop innovative vaccine strategies and novel drug targets for eradication of the disease.

Nitrogen metabolism plays a central role in bacterial physiology. It is linked to other metabolic networks

through the key molecules glutamine and glutamate. Glutamine acts as a nitrogen donor for many reactions in the cells, and is primarily synthesized from L-glutamate, ammonia and ATP by the enzyme glutamine synthetase (GS) (Reitzer, 1996; Fisher, 1999; Merrick & Edwards, 1995). In mycobacteria, GS and glutamate synthetase are the sole means of ammonia assimilation under nitrogen-limiting conditions.

Several mycobacterial species are known to possess multiple genetic loci that encode GS (Harper *et al.*, 2008; Amon *et al.*, 2009). Both *M. bovis* and *M. tuberculosis* have four different genes encoding GS (Cole *et al.*, 1998; Garnier *et al.*, 2003). In *M. tuberculosis*, the *glnA-1* gene encodes a class I GS (GSI) enzyme that is released into the culture medium and plays a crucial role in pathogenicity (Harth *et al.*, 1994), while the other three *glnA* genes (*glnA-2*, *glnA-3* and *glnA-4*) encode cytoplasmic proteins. All GSs are multimeric enzymes and catalyse synthesis of L-glutamine, while GlnA2 catalyses the synthesis of D-glutamine and isoglutamine. Only *glnA-1* has been shown to be essential for *M. tuberculosis* growth (Harth *et al.*, 2005). Disruption of *glnA-1* in *M. tuberculosis* results in glutamine auxotrophy, indicating its role in the synthesis of glutamine for the

Abbreviations: GS, glutamine synthetase; PLG, poly- α -L-glutamine; REMA, resazurin microplate assay.

A supplementary figure, showing the construction of the *M. bovis glnA-1* mutant, and two supplementary tables, showing bacterial strains and plasmids, and PCR primers, used in this study, are available with the online version of this paper.

bacillus (Tullius *et al.*, 2003). The genome of the saprophyte *Mycobacterium smegmatis* mc² 155 also contains homologues of *glnA*-1–4, in addition to several sequences that encode GSs similar to those present in soil-dwelling bacteria but absent from the mycobacterial genome (Amon *et al.*, 2009).

GS is implicated in providing glutamine for the synthesis of the poly- α -L-glutamine (PLG) layer associated with the cell wall of pathogenic mycobacteria, accounting for as much as 10 % of their weight (Wietzerbin *et al.*, 1975; Hirschfield *et al.*, 1990). It is absent in saprophytic and other non-pathogenic strains (Wietzerbin *et al.*, 1975). Treatment of *M. tuberculosis* with an inhibitor of GS, L-methionine-S-sulfoxamine, or with antisense oligonucleotides to *glnA*-1 mRNA, has been shown to inhibit PLG formation in the cell wall (Harth *et al.*, 2000). However, there is no direct evidence to demonstrate the involvement of *glnA*-1 in the synthesis of PLG and its role in fortifying the cell envelope of the pathogen.

M. bovis contains an array of genes involved in the uptake and synthesis of glutamine identical to that of *M. tuberculosis*; however, the role of different proteins has not been studied in this pathogen. Since *glnA*-1 is necessary for synthesis of glutamine, a key cell envelope constituent, its role in modulating cell wall chemistry needed to be investigated as a potential drug target against mycobacteria. This study is focused on examining the role of GS in the stress tolerance and cell wall resistance of *M. bovis*.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Supplementary Table S1. *M. bovis* strains were grown on Middlebrook 7H10 or 7H11 agar (Difco) containing 10 % (v/v) oleic acid, albumin, dextrose and catalase (OADC) (Becton Dickinson), 0.5 % (v/v) glycerol and 0.05 % (v/v) Tween 80, or in 7H9 broth (Difco) supplemented with 10 % (v/v) albumin, dextrose and catalase (ADC) (Becton Dickinson), 0.2 % (v/v) glycerol and 0.05 % (v/v) Tween 80, at 37 °C with shaking at 150 r.p.m. Hygromycin was used at a concentration of 50 μ g ml⁻¹ and kanamycin at 20 μ g ml⁻¹. *Escherichia coli* DH5 α was grown in Luria–Bertani medium with 100 μ g ampicillin ml⁻¹, 50 μ g kanamycin ml⁻¹ and 250 μ g hygromycin ml⁻¹.

Cloning, expression and *in vitro* disruption of the *glnA*-1 gene. The *glnA*-1 locus (Mb2244) with upstream promoter sequence was amplified from *M. bovis* genomic DNA by PCR using standard conditions. The amplified DNA was ligated into the pMV261 shuttle vector, producing pHCl, and electroporated into *M. smegmatis* mc² 155, as described by Pavelka & Jacobs (1999). The transformed *M. smegmatis* strain (*Ms-glnA1*) was selected on 7H10 agar plates containing 25 μ g kanamycin ml⁻¹ and tested for growth in THP-1 cells.

For *in vitro* disruption, the *glnA*-1 coding sequence was PCR-amplified as above. The purified 1.437 kb product was cloned into the pGEM-T Easy cloning vector, producing plasmid pHCl2. The ATP-binding domain of *glnA*-1 was deleted by inverse PCR (Pavelka & Jacobs, 1999) and a *KpnI* site was created, producing plasmid pHCl3. A 1.5 kb *aph* cassette with flanking *KpnI* sites from plasmid pHCl4 was excised and ligated into the *glnA*-1 coding sequence, producing pHCl5.

Generation of the *glnA*-1 mutant of *M. bovis*. A 2.8 kb *Bam*HI/*Spe*I fragment from plasmid pHCl5 was cloned into the temperature-sensitive vector pPR27 (Pelicic *et al.*, 1997), and the resulting construct (pHCl6) was electroporated into *M. bovis* as described earlier (Pavelka & Jacobs, 1999). The gentamicin-positive, single-crossover recombinants were grown to saturation with 10 mM glutamine, and the gentamicin-negative, sucrose-resistant phenotype was selected on 7H10 agar plates containing OADC, 0.2 % Casamino acid, 10 mM glutamine, 2 % sucrose and 20 μ g kanamycin ml⁻¹. Genomic DNA was isolated from the sucrose-resistant clones and allelic exchange was confirmed by Southern analysis. For complementation, the mutant was electroporated with pHCl7 containing the *glnA*-1 gene with a promoter in the pNBV1 vector (Howard *et al.*, 1995) and selected on 7H10 plates containing hygromycin.

Phenotypic characterization of the *glnA*-1 mutant of *M. bovis*.

A 50 ml volume of medium was inoculated with 10-day-old primary culture and incubated under shaking conditions at 37 °C. Growth characteristics were studied in 7H9 medium with enrichment or in Sauton's defined medium with 10 mM glutamine, and optical density was measured periodically. c.f.u. was also determined on supplemented 7H10 agar plates with or without glutamine.

Determination of the GS activity and protein profile of mycobacterial strains.

M. bovis cells were grown as above, and *M. smegmatis* was cultured in 7H9 medium with shaking at 37 °C. Extracellular GS activity was measured by the γ -glutamyl transfer reaction (Woolfolk *et al.*, 1966). Cultures of 10 ml volume were centrifuged at 8000 g for 15 min at 4 °C and washed once with PBS, and the pellets were resuspended in 5 ml PBS for sonication with a microtip. *M. smegmatis* was sonicated on ice for five cycles of 30 s each, and *M. bovis* for 15 cycles of 30 s each with intermittent cooling of the same duration, at 35 % amplitude. Cell debris was removed by centrifugation and the clear lysate was filtered through a 0.2 μ m pore-size Millipore filter. The culture filtrates were passed through a 0.22 μ m pore-size syringe filter and concentrated using a 30 kDa molecular weight cut-off Amicon filter (Millipore). GS activity was expressed as micromoles hydroxamate formed, determined from a standard curve obtained with authentic γ -glutamyl hydroxamate (Sigma).

The GS protein profile in the cellular and extracellular fractions of *M. smegmatis* strains was determined by SDS-PAGE followed by Western blotting with anti-GS and -GroEL antibodies.

Estimation of PLG in *M. bovis* and *M. smegmatis* strains. The strains were grown in 7H9 supplemented with 10 % ADC, 0.2 % (v/v) glycerol and 0.05 % (v/v) Tween 80 with shaking. The cell walls were prepared from 12–15-day-old *M. bovis* or 3-day-old *M. smegmatis* cultures, and PLG was purified as described elsewhere (Hirschfield *et al.*, 1990). The presence of PLG was confirmed by GC-MS analysis, after hydrolysis of the samples at 110 °C for 20 h with 6 M HCl followed by esterification with heptafluorobutyl isobutyl anhydride (MacKenzie & Hogge, 1977). GC-MS was performed using a Shimadzu GC-MS 2010, and an Rtx-5 MS capillary column (Restek), with an oven temperature range of 90–180 °C (5 min) at 4 °C min⁻¹, raised to 300 °C at 4 °C min⁻¹. The injection temperature was 280 °C, with an interface temperature of 290 °C. MS data were matched in the NIST05.LIB and WILEY8.LIB chemical libraries.

Uptake and intracellular growth of *M. bovis* and *M. smegmatis* strains in human THP-1 macrophages.

The human THP-1 macrophage monocytic cell line was maintained in RPMI 1640 medium containing 10 % heat-inactivated fetal bovine serum (FBS), 20 mM HEPES and 2 mM L-glutamine at 37 °C with 5 % CO₂/95 % air. Cells were seeded at 2 $\times$ 10⁵ cells per well in 24-well tissue-culture plates and differentiated with 20 nM phorbolmyristate acetate (PMA)

for 18 h. The efficiency of uptake by the phagocytic cells was determined at increasing bacteria:cell ratios (m.o.i.). For each data point, the mean of triplicate wells was used.

The monolayers were infected with washed, exponential-phase bacteria of *M. bovis* or *M. smegmatis* for 4 h at m.o.i. values of 5 and 50, respectively. After infection, the cells were treated with medium containing 20 µg gentamicin ml⁻¹ for a short time, and washed twice with RPMI medium. Fresh, complete medium was added and the plates were incubated at 37 °C. At different time points the cells were dislodged gently and centrifuged at 2000 r.p.m. for 3 min, washed two times with fresh RPMI medium, and lysed in sterile water. The lysate was diluted in 7H9 medium and plated on 7H11-OADC agar plates with or without 10 mM L-glutamine. The plates were incubated at 37 °C and colonies were scored after 4 days for *M. smegmatis* and 3–4 weeks for *M. bovis* strains. The morphology of the infected cells was observed under a phase-contrast microscope after different time periods, and viability was assessed by trypan blue exclusion (Theus *et al.*, 2004). The data at each time point are the mean from triplicate wells. The results presented are representative of multiple, independent experiments.

Response to mechanical and chemical stress. Twelve to fourteen day-old *M. bovis* cultures with an OD₆₀₀ ranging from 1.2 to 1.4 (~2 × 10⁷ c.f.u. ml⁻¹) were subjected to mechanical stress by sonication with seven cycles of 30 s duration at 25 % amplitude, with intermittent cooling of the same duration. In another experiment, the bacilli were treated with lysozyme at a concentration of 1 mg ml⁻¹ for 24 h and c.f.u. was determined for the treated and untreated samples by plating as described above.

Biofilm formation. For biofilm formation, 500 µl Sauton's medium in 24-well polystyrene tissue-culture plates was inoculated with 5 µl planktonic cultures (30 days old, OD₆₀₀ normalized to 1) and incubated for 3–4 weeks. Biofilm was quantified by removing the medium carefully and staining with 1 % crystal violet for 45 min. The wells were washed three times with water and air-dried. The dye was solubilized with 80 % ethanol and A₅₅₀ was measured (O'Toole *et al.*, 1999).

SDS sensitivity test. *M. bovis* strains were grown to exponential phase (15 days; OD₆₀₀ 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. Plates were incubated at 37 °C for 3–4 weeks and c.f.u. was recorded.

Drug sensitivity test. Drug sensitivity was tested by the resazurin microplate assay (REMA) (Palomino *et al.*, 2002) or by the agar dilution method (Hanson & Martin, 1978). Briefly, the drugs were prepared in either DMSO or sterile deionized water, and twofold serial dilutions were made in 7H9 growth medium in microtitre plates. The drug concentrations ranged from 0.003 to 1 µg ml⁻¹ for isoniazid, 0.0075 to 2 µg ml⁻¹ for ethambutol, 0.00007 to 0.018 µg ml⁻¹ for rifampicin, and 0.39 to 100 µg ml⁻¹ for D-cycloserine. Approximately 5 × 10⁴ c.f.u. of a 12-day-old *M. bovis* culture was added per well; control wells contained only bacteria and growth medium. The plates were incubated at 37 °C for 7 days and 0.02 % resazurin was added. The plates were observed after 48 h for a colour change from blue to pink. The visual MIC was determined as the lowest concentration of the drug at which a colour change was prevented. In the agar dilution method, 10 µl culture was spotted onto an agar plate containing the drug and incubated at 37 °C for 3–4 weeks. The MIC was recorded as the concentration at which bacterial growth was completely inhibited.

Virulence in BALB/c mice. Six-week-old male BALB/c mice (18–20 g) were challenged intravenously through the lateral tail vein with

exponential-phase cultures of *M. bovis* strains. Animals were weighed twice monthly and checked daily for clinical symptoms of weight loss and lack of response to stimuli, as displayed in rodent tuberculosis. The mice (*n*=3) from each group were sacrificed at 2, 4, 6 and 15 weeks, and the lungs and spleens were cultured individually for c.f.u. determination on 7H11 plates with or without 10 mM L-glutamine. Colonies were scored after 3–4 weeks incubation at 37 °C. Animal research was conducted in compliance with animal ethics committee and tuberculosis challenge facility guidelines.

RESULTS

Construction and characterization of the *M. bovis* *glnA-1* mutant

Supplementary Fig. S1(a) shows a schematic map of the insertional inactivation of the *glnA-1* locus. Genomic DNA from two sucrose- and kanamycin-resistant colonies reacted with the labelled probe in a Southern blot after *Sma*I digestion. A ~3.8 kb band was observed in the mutant as compared with a 2.3 kb fragment in the wild-type (Supplementary Fig. S1b), indicating allelic exchange of the wild-type gene by the disrupted copy; colony 1 was used for further characterization.

Growth characteristics

Growth of the mutant in 7H9 broth with enrichment showed a longer lag phase than the wild-type strain (Fig. 1a, c), but later cell densities and c.f.u. values were not very different in the two strains, indicating that the *glnA-1* strain was able to acquire nitrogen from other sources in the medium [L-glutamic acid, 3.4 mM; ammonium sulphate, 3.8 mM; ferric ammonium citrate, 0.14 mM; and enrichment containing 10 % (v/v) albumin]. In contrast to *M. tuberculosis*, the *M. bovis* *glnA-1* mutant replicated for 3–4 logs in the rich medium with no L-glutamine addition. However, in Sauton's defined medium, no growth was observed in the absence of glutamine. The growth defect was abolished in the presence of 10 mM glutamine (data not shown).

GS activity and PLG content in the cell wall

Under aerobic conditions, the GS activity peaked on day 10 in *M. bovis* strains. The wild-type contained twofold higher intracellular GS activity compared with the mutant (Fig. 1b); the activity was restored to the wild-type level in the complemented strain, indicating the direct involvement of the *glnA-1* gene in total cellular GS activity. The extracellular activity was proportionately reduced in both strains: the wild-type had about one-sixth of the intracellular activity, while the mutant showed only a basal level of activity which was similar to that of the medium alone (Fig. 1d). Like the enzyme activity, the PLG content of the cell wall was also drastically reduced in the mutant (below detectable limits) compared with ~4 and 5 % of the dry weight of the cell wall in the wild-type and complemented strains, respectively.

Fig. 1. Growth and enzyme activity of the *glnA-1* mutant in broth culture. (a) The *glnA-1* mutant (■) and wild-type *M. bovis* (▲) were inoculated to an initial optical density of 0.004–0.005 in enriched 7H9 medium. (a) OD₆₀₀, (c) c.f.u., (b and d) intra- and extracellular GS activity, respectively. *glnA-1* mutant (■), wild-type *M. bovis* (▲) and complemented *glnA-1* (●). Data are mean ± SD of values obtained from three independent cultures.

Control *M. smegmatis* cells (containing pMV261) produced a very low level of endogenous GS protein compared with cells expressing the *glnA-1* gene episomally (Fig. 2a, b). Most of the catalytically active GS (85%) was released extracellularly by *Ms-glnA1*, in contrast to wild-type *M. bovis*, in which only ~14% of the endogenous GS was detected in the culture supernatant (Fig. 2a). The GS protein was detected in the culture medium of the *Ms-*

glnA1 strain after 24 h, probably exported by a specific mechanism and not by cell lysis, as an intracellular protein, GroEL, though produced in the cell, was not detected (Fig. 2b). The PLG content of *Ms-glnA1* was found to be 10–12% of the cell wall dry weight, which was more than twice the amount obtained from native *M. bovis* and could be due to expression from a multicopy plasmid. The control vector-transformed cells produced no PLG. These results

Fig. 2. Protein expression and GS activity in *M. smegmatis* strains. (a) Enzyme activity in the cell lysate and culture supernatant fractions at 48 h. (b) Western blots of the fractions analysed by anti-GS antibodies. M, prestained protein markers. (i) Lanes: 1, 3 and 5, cell lysate of *Ms-glnA1*; 2, 4 and 6, wild-type. (ii) Lanes: 1–4, culture supernatant of *Ms-glnA1* at 15, 24, 48 and 56 h, respectively; 5 and 6, wild-type strain at 48 and 56 h, respectively. (iii) Blotting with anti-GroEL antibodies: lanes 1 and 2, cell lysates of *Ms-glnA1* and wild-type, respectively, at 48 h; lanes 3, 4 and 5, culture supernatants of *Ms-glnA1* at 24, 48 and 56 h, respectively; lanes 6, 7 and 8, wild-type at 24, 48 and 56 h respectively.

demonstrate the involvement of the *glnA-1* gene in cell wall-associated PLG synthesis.

Intracellular growth of *M. bovis* and *M. smegmatis* strains in human THP-1 cells

To examine whether the altered cell wall chemistry played a role in the uptake of the mutant by THP-1 macrophages, the m.o.i. values of the mutant and the wild-type were compared. To achieve internalization of an average of two to six bacteria per macrophage, a higher m.o.i. was required for the mutant than for the wild-type (Fig. 3a), suggesting alterations in the cell surface. When cells were infected with wild-type *M. bovis*, the viability of the cells was reduced to 51 % in 48 h, as determined by trypan blue exclusion; the *glnA-1* mutant caused less damage to the cells (81 % viability), reflecting the reduced virulence of the strain. The complemented strain restored the wild-type phenotype, reducing cellular viability to 49 % (Fig. 3b).

The mutant showed an attenuated phenotype in macrophages cultured in standard RPMI medium (2 mM glutamine), showing a reduction of more than 1 log in

Fig. 3. Uptake of *M. bovis* strains and viability of infected THP-1 cells. (a) Macrophages were infected and the number of internalized bacteria was determined by plating at time zero. Black bars, wild-type *M. bovis*; white bars, *glnA-1* mutant. (b) Cellular viability by trypan blue exclusion. Black bars, control uninfected cells; hatched bars, wild-type *M. bovis*; grey bars, *glnA-1* mutant; white bars, *glnA-1*⁺ complemented mutant. Data are mean $\pm$ SEM of triplicate wells and are representative of three independent experiments.

Fig. 4. Intracellular growth of *M. bovis* and *M. smegmatis* strains in THP-1 cells. Cells were cultured in RPMI medium containing 2 mM L-glutamine. (a) Solid lines: (▲) wild-type, (●) *glnA*⁺ complemented, (■) *glnA-1* mutant. The mutant was also grown in cells cultured in RPMI with 5 mM (dotted line) and 10 mM (dashed line) L-glutamine. (b) (●) *M. smegmatis*, (■) *M. smegmatis* containing vector pMV261, (▲) *Ms-glnA1*. The data presented are mean $\pm$ SEM of triplicate wells and are representative of three individual experiments.

c.f.u. after 72 h, whereas the wild-type strain multiplied, showing an increase of more than 2 logs after 72 h. Complementation of the mutant with *glnA-1* in a plasmid restored the growth defect of the mutant (Fig. 4a). When the tissue-culture medium was supplemented with 5 and 10 mM glutamine, the c.f.u. of the mutant strain increased by $\sim$ 1 and 2 logs, respectively, at 72 h, indicating its dependence on extracellular glutamine *in vivo* (Fig. 4a). A higher glutamine concentration of 20 mM was inhibitory to the bacteria (data not shown).

Ms-glnA1 survived in the THP-1 cells longer, to 72 h ($P < 0.001$), than the untransformed or vector-transformed control cells (Fig. 4b). In the control group, an increase of $\sim$ 1 log occurred at 24 h, after which the number of c.f.u. declined. In contrast, *M. smegmatis* cells containing heterologously expressed *glnA-1* continued to replicate up to 72 h within the macrophages, showing the necessity of GS for intracellular survival.

Fig. 5. Sonication and lysozyme sensitivity of the *glnA-1* mutant. (a) Cells were treated with lysozyme at a concentration of 1 mg ml⁻¹ for 24 h and plated for c.f.u. determination. (b) Cultures were sonicated and plated; black bars, 12-day-old cultures; white bars, 25-day-old cultures. Error bars, SD from three independent experiments.

Cell wall sensitivity of the *glnA-1* mutant

The cell wall of the mutant was highly susceptible to physical and chemical stresses. Sonication of a 12–15-day-old culture

resulted in a 62 % reduction in c.f.u. of the mutant in comparison with a 22 % reduction in the wild-type strain, corresponding to an approximately threefold increase in the sensitivity of the mutant (Fig. 5a). At a later time point (30 days), the mutant cells became somewhat more resistant to sonication (data not shown). The complemented strain behaved similarly to the wild-type. Likewise, the mutant was more sensitive to lysozyme treatment, showing a four- to fivefold increase in sensitivity compared with the wild-type or the complemented strain, in both the exponential and stationary phases of growth (Fig. 5b). The SDS tolerance of the mutant was also reduced drastically compared with the wild-type strain, and the defect was abolished in the complemented strain, reflecting alterations in the chemistry of the cell wall (Fig. 6).

Effect on biofilm formation

The mutant was defective in forming biofilms on a polystyrene surface. The mutant cells showed an ~50 % reduction in biofilm formation, while complementation restored the defect to a large extent, reiterating the involvement of *glnA-1* in modulating the cell surface properties of mycobacteria (Fig. 7).

Drug susceptibility test

MIC values for various drugs are shown in Table 1. In the *glnA-1* strain, the MIC values for rifampicin and D-cycloserine were reduced about eightfold compared with the wild-type strain. The MIC values for ethambutol and isoniazid remained unchanged in the mutant; however, in the *glnA-1* complemented strain, the two drugs had two- and fourfold higher MICs than the wild-type strain, which could be due to expression of the protein in higher copy number from the plasmid, causing a higher deposition of PLG in the cell wall.

Fig. 6. SDS sensitivity of the *glnA-1* mutant. Dilutions of 12-day-old bacterial cultures were spotted onto 7H10 agar plates with (+ SDS) or without (-SDS) 0.01 % SDS and incubated at 37 °C for 3–4 weeks. (a) Undiluted culture, (b) 1 : 10 diluted culture.

Fig. 7. Biofilm formation by *M. bovis* strains. Stationary phase planktonic cultures were inoculated in Sauton's medium containing 10 mM L-glutamine and incubated for 4 weeks. Adherent biofilm was stained with 1% crystal violet and A_{550} was measured. Error bars, SEM from triplicate wells. The experiment was repeated three times with similar results. Control, medium only.

Replication in BALB/c mice

The ability of *M. bovis* strains to survive and replicate in the spleen and lungs of BALB/c mice was followed for up to 15 weeks. The c.f.u. counts on day 0 of the three cultures were wild-type 1×10^5 , *glnA-1* mutant 1.7×10^5 and complemented strain 1.4×10^5 . At 2 weeks post-injection, the number of c.f.u. dropped slightly in all the groups. After 4 weeks, the number of mutant cells was reduced to $\sim 10^2$ in the organs (Fig. 8a), and at 6 weeks no colonies were obtained (Fig. 8b). In contrast, both the wild-type and complemented strains infected lungs and spleen, and after an initial fall, the bacterial burden increased by 2–3 logs at 15 weeks (Fig. 8c). No deaths were recorded until 19 weeks in any group.

Table 1. MIC values of antitubercular drugs for *M. bovis* strains, determined by REMA

Each concentration of drug was tested in triplicate. The data were obtained from three independent experiments and similar results were obtained each time.

Drug	MIC* ($\mu\text{g ml}^{-1}$)		
	<i>M. bovis</i>	<i>glnA-1</i> mutant	<i>glnA-1</i> ⁺
Isoniazid	0.06	0.06	0.25
Ethambutol	0.5	0.5	1
Rifampicin	0.002	0.00031	0.002
D-Cycloserine	25	6.25	25

*MIC was defined as the lowest concentration of the drug that prevented a change in colour from blue to pink.

Fig. 8. Growth of *M. bovis* strains in BALB/c mice. Each group containing three mice was infected through the lateral tail vein with a dose of $1-2 \times 10^5$ c.f.u. of each *M. bovis* strain. c.f.u. values are shown at (a) 4 weeks, (b) 6 weeks and (c) 15 weeks. Lungs and spleens from each mouse were homogenized and dilutions were plated. Black bars, lungs; white bars, spleen. Data are the mean $\pm$ SD from three individual mice, and are representative of two independent experiments.

DISCUSSION

The inherent resistance of mycobacteria to common antibiotics and stressful conditions in the host is largely attributed to the unique chemistry of their cell walls (Hett & Rubin, 2008; Vandal *et al.*, 2009). Several earlier studies have reported the presence of poly-L-glutamate/PLG in the cell wall of pathogenic strains (Wietzerbin *et al.*, 1975; Hirschfield *et al.*, 1990; Harth & Horwitz, 1999); however, very little is known about its physiological role. Since PLG is associated with the cell wall, it is likely to influence the cell envelope chemistry and consequently the response of the pathogen to various stress factors.

The association of PLG exclusively with pathogenic species emphasizes its importance in mycobacterial pathogenesis. Other pathogenic Gram-positive bacilli, such as *Bacillus anthracis* and *Staphylococcus epidermidis*, produce a poly- γ -glutamate capsule on the cell surface as a virulence factor (Candela & Fouet, 2006). Apparently, the loss of PLG decreases the mechanical strength of the *M. bovis* cell wall and increases its sensitivity to an important effector of innate immunity, lysozyme, resulting in faster clearance of the mutant from THP-1 cells. In view of the greater resistance of mycobacterial peptidoglycan (PG) to lysozyme (Hett & Rubin, 2008), the higher sensitivity of the *glnA-1* mutant to the latter demonstrates the participation of PLG in protecting the murein layer, either by reducing its accessibility to the enzyme or by chemically fortifying the PG sacculus against enzyme attack. Further, the increased sensitivity of the *glnA-1* mutant to SDS also supports the role of the SDS-insoluble PLG in cell wall protection.

The defect in biofilm formation could be due to the changed surface chemistry of the mutant. The ability to form biofilms is an important virulence determinant of pathogenic mycobacteria (Ojha *et al.*, 2008). The biofilm formed by *M. tuberculosis* is known to contain abundant free mycolic acid (Ojha *et al.*, 2005, 2008); at this stage we do not know whether mycolic acid synthesis or its content in the cell wall of the mutant is affected. Other genetic loci identified in biofilm formation in mycobacteria are linked to enzymes of the tricarboxylic acid (TCA) cycle, glycolipid biosynthesis (Yamazaki *et al.*, 2006) and extracellular GroEL1 (Ojha *et al.*, 2005). Moreover, how GS influences biofilm formation in *M. bovis* needs further investigation.

The greater susceptibility of the mutant to the lipophilic antitubercular drugs rifampicin and D-cycloserine was expected, and was more likely due to an increased permeability of the cell wall and not a direct effect on the targets, the β -subunit of RNA polymerase and D-Ala-D-Ala ligase, respectively. This also explains the unchanged MICs observed for the non-lipophilic antibiotics isoniazid and ethambutol, which also act on cell wall targets.

The physiological role of *glnA-1* in sustaining bacterial growth and survival within the host cells is reiterated by the increased mean survival time of the *Ms-glnA1* strain in THP-1 cells. It is intriguing that *M. smegmatis*, despite four homologues (*glnA1-4*) in the genome, does not produce a GS protein, and that the presence of the *M. bovis glnA-1* locus leads to synthesis of PLG in *M. smegmatis*. A comparative analysis of the two loci may reveal interesting information about the gene expression of pathogenic mycobacteria. It has also been postulated that GS, by assimilation of ammonium ions into amino acids as glutamate and glutamine (Harper *et al.*, 2008; Amon *et al.*, 2009; Merrick & Edwards, 1995), helps in the removal of excess, toxic ammonia from the intraphagosomal milieu, thereby promoting the survival of the pathogen; however, this possibility needs to be examined

experimentally. Based on our results, it may be argued that in addition to alterations in bacterial metabolism, the chemical changes brought about in the cell wall due to the absence of PLG play a significant role in the attenuation of *M. bovis*.

The diverse phenotypic changes caused by disruption of the *glnA-1* gene provide a basis for using it as a drug target as well as a vaccine candidate. However, the utility of GS as a drug target is somewhat limited due to its similarity to the mammalian enzyme in the relative placement and orientation of all the substrates and cofactors, except the nucleotide-binding site (Krajewski *et al.*, 2008). The pathway of PLG synthesis in mycobacteria is not known; whether it is synthesized intracellularly and carried to the surface or assembled extracellularly, the enzymes involved and the specific role(s) of *glnA-1* need to be established to identify potential new drug targets against tuberculosis.

In conclusion, although the roles of multiple GSs of mycobacteria have been investigated extensively in the past, surprisingly, none of the studies was focused on the impact of PLG depletion from the cell wall. Our data for the first time reveal the importance of this cell wall polymer in enhancing the stress tolerance of the pathogen and open new possibilities for the development of antitubercular drugs.

ACKNOWLEDGEMENTS

This work was supported by the Department of Biotechnology (DBT), India. We are grateful to Dr V. Pelicic, Imperial College London, UK, for providing the allelic exchange vector pPR27 and Dr M. A. Horwitz, UCLA School of Medicine, Los Angeles, CA, USA, for the plasmid DNA pNBV1. GC-MS analysis was done at the Advanced Instrumentation Facility, Jawaharlal Nehru University. All the animal experiments and *Mycobacterium* culture work were conducted at the Tuberculosis Aerosol Challenge Facility (TACF) laboratory, International Centre for Genetic Engineering and Biotechnology.

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Edited by: M. Daffé