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Research paper

The *lpqS* knockout mutant of *Mycobacterium tuberculosis* is attenuated in Macrophages

Suba Sakthi, Sujatha Narayanan*

Department of Immunology, National Institute for Research in Tuberculosis, (Formerly Tuberculosis Research Centre), Chennai, India

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ABSTRACT

Lipoproteins of *Mycobacterium tuberculosis* (*M. tuberculosis*) represent an important class of cell envelop proteins. On the whole, 99 putative lipoproteins have been identified in the genome of *M. tuberculosis*. Earlier investigations on individual mycobacterial lipoproteins demonstrate that some lipoproteins elicit a strong immune response, while others are virulence factors in different models of infection. LpqS is an uncharacterized lipoprotein encoded by the open reading frame Rv0847 of *M. tuberculosis*. In the present study, we have characterized this putative lipoprotein LpqS with respect to the virulence of *M. tuberculosis*. A mutant of the *M. tuberculosis* H_{37}Rv strain not producing LpqS (Δ*lpqS*) was generated by specialized transduction. The deletion mutant showed reduced growth in Sauton's minimal media and was highly sensitive to SDS and copper, compared to the wild type when grown on solid media. *In vitro* infection studies showed that the mutant was attenuated for growth in PMA-activated THP-1 cells. Complementation of the mutant with a single copy of the gene cloned under the hsp60 promoter partially restored the phenotype of the wild type strain H_{37}Rv. Thus *lpqS* plays an important role in sensing the host macrophage environment and might be required for the intracellular survival of *M. tuberculosis*. Cotranscription of *lpqS* with the genes downstream *cysK2*, Rv0849 and Rv0850 was also demonstrated by Reverse transcription PCR (RT-PCR) of intergenic regions.

1. Introduction

Tuberculosis (TB) has been a global threat for several years and is still one of the biggest killers among the infectious diseases. In the year 2010, WHO reported nearly 8 million new cases of TB and 1.4 million deaths which included 350,000 people with HIV. Despite the use of live attenuated vaccines and several antibiotics, the main glitch that unables the eradication of the disease is the ability of the tubercle bacilli to establish a clinically asymptomatic long term infection in the human host which can later manifest as acute or chronic disease. Hence a better understanding of the biology of *Mycobacterium tuberculosis* and the virulence determinants associated with its pathogenesis is required to develop newer vaccines and antitubercular drugs to eliminate this worldwide epidemic.

The complete genome sequencing of *M. tuberculosis* is a major breakthrough in this line of interest (Cole et al. 1998). Whole genome sequencing has identified nearly 100 ORFs encoding putative lipoproteins (Camus et al. 2002; Sutcliffe and Harrington 2004). The presence of such lipidated proteins contributes much to the exceptionally less permeable cell envelop of *M. tuberculosis*, protecting it against several stress conditions. Lipoproteins also represent a significant class of cell envelope proteins ideally positioned for interactions between the organism and the host. Despite all this, very few mycobacterial lipoproteins have been characterized based on their potential implications in pathogenesis and immunogenicity.

Previous studies using purified mycobacterial lipoproteins on host macrophages and dendritic cells (Post et al. 2001; Gehring et al. 2004; Pai et al. 2004; Pecora et al. 2006) and several vaccination studies (Yeremeev et al. 2000; Hovav et al. 2003; Rao et al. 2005; Mir et al. 2009) have clearly demonstrated lipoproteins as potent TLR2 agonist possessing immunomodulatory functions. Adhered to the surface of the cell wall, lipoproteins were identified to play a crucial role in host-pathogen interaction such as initiating inflammatory responses, functioning as accessory proteins modulating the activity of two component systems (Nguyen et al. 2010.) and as transporters contributing to virulence (Sulzenbacher et al. 2006). Lipoproteins like pstS3 and pstS1 are identified as very good vaccine candidates. LprG, LpqH and LprA are antigenic lipoproteins also inducing homeostatic down-regulatory mechanisms that limit potentially damaging responses (Gehring et al. 2004; Pai et al. 2004; Rao et al. 2005; Pecora et al. 2006).

* Corresponding author at: Department of Immunology, National Institute for Research in Tuberculosis, (Formerly Tuberculosis Research Centre), Indian Council of Medical Research, Mayor V.R. Ramanathan Road, Chetpet, Chennai 600 031, India. Tel.: +91 44 28369727; fax: +91 44 28362528.
E-mail address: sujatha.sujatha36@gmail.com (S. Narayanan).

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Lipoproteins are generally characterized by the presence of an N-terminal signal peptide. LpqS is a cell membrane associated lipoprotein of *M. tuberculosis* and is highly conserved among slow growing mycobacteria. Unlike lipoproteins LprG, LprA, LprF and LppX, (LppX/LprAFG family of lipoproteins), LpqS shares no sequence homology with other lipoproteins. Previous studies have implicated LpqS in the survival of *M. tuberculosis* under non-replicating conditions and also in enduring hypoxic response (Muttucumaru et al. 2004; Gonzalo-Asensio et al. 2008). In addition *lpqS* lies within a region of *M. tuberculosis* genome comprising a multi-copper oxidase Rv0846c and a non-specific metal permease Rv0849. This region is identified to be a member of the copper inducible five-locus regulon specific to virulent mycobacterial species. The entire five locus regulon is controlled by the copper-sensing repressor RicR (regulated in copper repressor) (Festa et al. 2011). In view of these findings, we hypothesized that *lpqS* might play a role in the intracellular survival of *M. tuberculosis* and might be essential to combating copper toxicity. This study is focused mainly on gaining an insight into the role of *lpqS* in the growth and survival of *M. tuberculosis*. Here we report the construction of the first *lpqS* mutant of *M. tuberculosis* H_{37}Rv. Our experiments showed that the gene deletion mutant was defective in growth under increased copper concentrations and was more sensitive to SDS *in vitro*. The mutant was also found to be attenuated for growth in THP1-derived macrophages. Furthermore, RT-PCR analysis carried out with the RNA samples isolated from copper-induced cultures of *M. tuberculosis* H_{37}Rv provides evidence that the gene *lpqS* and the three genes downstream (*cysK2*, Rv0849 and Rv0850) exist as a part of an operon, regulon or are controlled by a common regulator.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

The *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB agar (Difco) for plasmid isolation, isolation of recombinant clones and transformation. LB broth is supplemented with 10 mM $MgSO_4$ and 0.2% maltose for transduction with λ packaged cosmids. *Mycobacterium smegmatis* (*M. smegmatis*) mc^2155 was grown in LB broth containing 0.5% Tween 80 (LBT). Mycobacterial strains other than *M. smegmatis* were grown in 7H9 (Difco) medium supplemented with 10% ADS (Albumin dextrose saline) and 0.05% Tween80. Antibiotics were used at prescribed concentration (kanamycin (25 μg/ml for Mycobacteria and 50 μg/ml for *E. coli*), carbenicillin (50 μg/ml) and hygromycinB (150 μg/ml for *E. coli* and 50 μg/ml *M. smegmatis*) when required. Propagation of mycobacteriophages in *M. smegmatis* mc^2155 was carried out using basal 7H9 medium supplemented with 0.4% glycerol as bottom agar and top agar containing 0.6% agar in 7H9 media supplemented with 0.2% dextrose. Gene sequences for primer designing was retrieved from Tuberculist and primers procured from Ocimum Biosolutions. The primers used for knockout study are listed in Table 1 and primers used for RT-PCR experiments are listed in Table 3. The plasmids used and generated in this study are given in Table 2.

2.2. Construction of M. tuberculosis LpqS knockout mutants

LpqS gene disruption was carried out following the protocol of Bardarov et al. (2002). Recombinant cosmid containing the allelic exchange substrate (AES) was constructed by amplifying the DNA segments flanking the 5′ and 3′ regions of the *lpqS* gene (named left arm and right arm) using suitable primers (Table 1). The flanking regions were then cloned into the suicidal vector p0004S directionally, on either side of the hygromycin resistance-*sacB* gene cassette (3.6 Kb). The resulting recombinant cosmid p0847S contained the AES needed to construct the bacteriophage. The *lpqS* gene disrupted by the selectable marker was then cloned into the temperature-sensitive TM4 shuttle phasmid phAE159 to generate the specialized transducing mycobacteriophage. High titer phages were then prepared at a replication permissive temperature of 30 °C and gene was deleted from *M. tuberculosis* H_{37}Rv by specialized transduction. Allelic exchange occured as a result of a double crossover between the homologous DNA arms flanking the disrupted gene. Transductants were then screened for gene disruption by PCR using forward primer for hygromycin resistance gene (Hyg^R) and right arm reverse primer (RR) (Fig S1). Gene deletion was then confirmed by southern blotting using radioactively labeled right arm as the probe (Fig S1). The *lpqs::hyg* strain was designated Δ*lpqS*. To complement the gene-disrupted strain, *lpqS* coding region was cloned under the hsp60 promoter of the pMV361 (integrative backbone) vector (Stover et al. 1991) and the construct was named pSR1. Integration of the gene was achieved by electroporating the Δ*lpqS* strain with the construct pSR1 and selecting the gene complemented colonies on plates containing hygromycin and kanamycin. Complementation was then confirmed by PCR using *lpqS* primers and the complemented strain designated as CΔ*lpqS*.

2.3. In vitro growth kinetics of the lpqS disrupted mutant

Logarithmic phase cultures of the wild type H_{37}Rv, Δ*lpqS* mutant and the complemented strain CΔ*lpq*S were washed thrice and diluted to 0.05 OD_{600} in enriched Middlebrook 7H9 media and also in Sauton's minimal media both supplemented with 0.05% Tween80. Diluted cultures were then grown in the shaker incubator at 200 rpm and at 37 °C. Aliquots of the cultures were withdrawn at regular intervals on day 2, 4, 7, 10, 14 and 21 and the growth monitored by plating serially diluted cultures on the 7H10-ADS plates at specified time points. CFU measurements were made after incubation of the plates at 37 °C for 4–5 weeks.

2.4. Sensitivity to SDS

With LpqS being highly expressed under hypoxic conditions, the ability of the mutant to grow under surfactant stress was compared with both the wild type and complemented strain. Serial dilutions of the logarithmic phase cultures of wild type H_{37}Rv, Δ*lpqS* and the complemented strains were plated on plain 7H10-ADS plates and 7H10-ADS plates supplemented with SDS at concentrations 0.01% and 0.001%. The growth was recorded after 4 weeks of incubation at 37 °C.

2.5. Biofilm formation and colony morphology

LpqS being predicted as a membrane associated protein, the ability of the *lpqS* mutant to form biofilm was compared with the wild type and the complemented strain. Biofilm formation was assessed by growing the strains in polystyrene petriplates containing Sauton's minimal media without Tween for 4–6 weeks at 37 °C. In addition wild type, mutant and complemented strain cultures were serially diluted, plated on 7H10 plates and incubated at 37 °C for 4–6 weeks. Plates were then observed for difference in colony morphology.

2.6. Sensitivity to copper, zinc and iron

LpqS was earlier identified as a member of copper inducible five locus regulon and is predicted to play a role in copper export. Hence the ability of the Δ*lpqS* mutant strain to grow at higher copper concentrations was compared with wild type by plating 10-fold serially diluted logarithmic phase cultures of the wild type, mutant and

Table 1
Primers used in this study.

Primers	Description	Sequence
lpqS LL	Forward primer to amplify left arm of *lpqS*	5′TTTTTTTTCCATAAATTGG GTCGATTGCAGCGGTCATCC 3′
lpqS LR	Reverse primer to amplify left arm of *lpqS*	5′TTTTTTTTCCATTTCTTGG CACATGACGGTGGAGCTGGG 3′
lpqS RL	Forward primer to amplify right arm of *lpqS*	5′TTTTTTTTCACAGAGTGGTTCGCACCGGTCAAGACCT-3′
lpqS RR	Reverse primer to amplify right arm of *lpqS*	5′ TTTTTTTTCACCTTGTGGTCAGCATGCGCGCGATGAT-3′
Hyg^R	Forward primer to amplify hygromycin resistance gene	5′GCCTGGTGCAACTGCATCTC3′
lpqS HindIII fwd	Forward primer to amplify *lpqS* for pMV361 cloning	5′CCAAGCTTGGGTGGTGTGGATGCGATCGG -3′
lpqS ClaI rev	Reverse primer to amplify *lpqS*	5′CCATCGATGGTCAGCGACGAGCCAGGCAGAAC 3′

Table 2
Plasmids used in this study.

Plasmids	Description	Source
p0004S	Allelic exchange vector carrying Hyg^R-Sac cassette	William R Jacobs Jr (Unpublished)
p0847S	Suicide recombination delivery vector carrying hyg^r–sacB for disrupting *lpqS* in *M. tuberculosis* H_{37}Rv	This study
Phae159	Conditionally replicating shuttle phasmid vector	William R Jacobs Jr (Unpublished)
pMV361	Integration-proficient *E. coli-Mycobacterium* shuttle vector	Stover et al. (1991)
pSR1	pMV361 derivative harboring *lpqs* under the Hsp60 promoter for the complementation of the Δ*lpqS* mutant	This study

the complemented strain in 7H10-ADS plates incorporated with varying copper concentrations (25, 50, 75 and 100 μM). Furthermore the growth of the mutant was also assessed under conditions unrelated to copper by growing on solid media containing 100 μM of iron and 0.05% zinc. Simulataneously suitable controls were also maintained. The growth was recorded after 4 weeks of incubation at 37 °C.

2.7. Intracellular viability of the mutant

Intracellular viability of the *lpqS* knockout mutant was determined using macrophage infection studies. THP1 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS). The cells were then seeded onto 24 well plates (1million cells/well) and differentiated into macrophages using 50 mM phorbol 12-myristate 13-acetate (PMA). Cells were incubated overnight, washed twice with RPMI medium and left resting for two days. Macrophages were then infected in triplicates with wild type H_{37}Rv, Δ*lpqS* mutant and the complemented strain at an MOI of 1:10. Phagocytosis was allowed to take place for 4 h, after which monolayers were washed with RPMI to remove the non-phagocytosed bacilli. The infected macrophages were then incubated with fresh RPMI supplemented with 10% FBS at 37 °C in the presence of 5% CO_2. Intracellular bacilli were then recovered by lysing infected macrophages with 1% ice-cold trypsin on day 0, 1, 3, 5 and 7 post infection. Bacterial load was then estimated by plating serial dilutions of the lysates onto Middlebrook 7H10 plates and counting CFUs after 4 weeks of incubation at 37 °C. Cell-free culture supernatants were collected and stored at −80 °C for cytokine analysis.

2.8. Cytokine analysis by ELISA

Cell-free culture supernatants collected from the infected cell lines (THP-1 derived macrophage cell lines infected with H_{37}Rv, Δ*lpqS* mutant and the complemented strain CΔ*lpqS*) at different time points were analyzed for the levels of secreted cytokines TNF-α, IL-12, and IL-6 by sandwich ELISA using BD optEIA ELISA kit (BD pharmingen, USA) as per the manufacturers protocol.

2.9. Isolation of total RNA from M. tuberculosis

Cells of a 50 ml *M. tuberculosis* culture was resuspended in Trizol (Invitrogen) and disrupted with 0.1 mm zirconium beads in a mini bead beater. The total RNA was purified using an RNeasy purification kit (Qiagen). RNA samples were treated with RNase free DNaseI to remove contaminating DNA. The first strand cDNA was synthesized from 1 μg of total RNA using Quantitect Reverse transcriptase kit (Qiagen).

2.10. Statistics

All data are representative of three independent experiments carried out using biological replicates. Two way ANOVA was used to analyze the data obtained from ELISA and sensitivity assays carried out using SDS and the metal ions copper, zinc and iron. ($^{**}P < 0.01$ was said to be significant and $^{***}P < 0.001$ was said to be highly significant).

3. Results

3.1. Bioinformatic analysis

The sequence of *M. tuberculosis lpqS* was retrieved from Tuberculist and used as a query sequence for a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast). Type I signal peptides were identified using LipoP 1.0 (http://www.cbs.dtu.dk/services/LipoP/) and signal P 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). Sequences for *lpqS* of other members of mycobacteria were obtained from TIGR database (www.tigr.org). The sequence

Table 3
Primers used for RT-PCR experiments.

Intergenic region no.	Forward primer	Reverse primer
1	5′-GTTCTGCCTGGCTCGTCGCT-3′	5′-GTTGGAGGTGATGCTTGTTG-3′
2	5′-TTCGCACCGGTCAAGACCTG-3′	5′-AGTGACTTGATATCCCTCCG-3′
3	5′-ACACGCAGCACCACGGTGAT-3′	5′-AAATTGCGCACGCCCATCAC-3′
4	5′- CATCTACAACGACGCGTACT-3′	5′-AAATTGCGCACGCCCATCAC-3′
5	5′-CGTCGGTTGGACACATTCAC-3′	5′-TCCTGATTGAATACCGCACG-3′
6	5′-ACACGCAGCACCACGGTGAT-3′	5′-TCCTGATTGAATACCGCACG-3′
7	5′-GTTCTGCCTGGCTCGTCGCT-3′	5′-AAATTGCGCACGCCCATCAC-3′

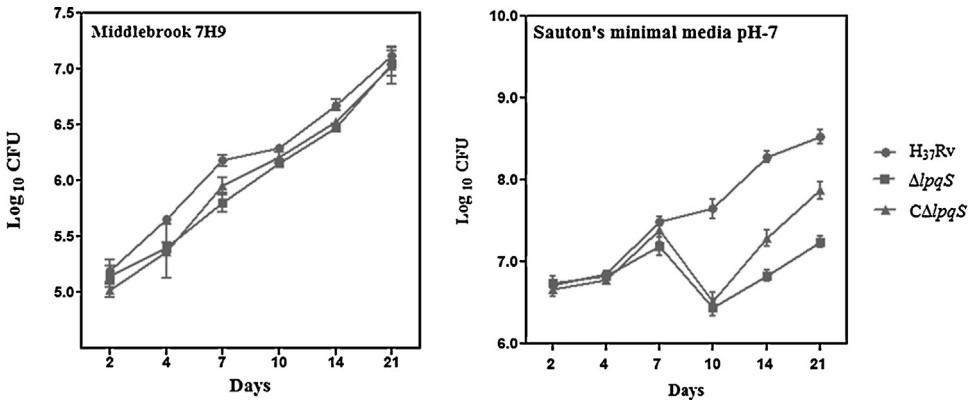

Fig. 1. *In vitro* growth analysis of *M. tuberculosis* Δ*lpqS*. Wild type H_{37}Rv, Δ*lpqS* and CΔ*lpqS* were grown in enriched Middlebrook 7H9 media and Sauton's minimal media at pH 7.0 under standard culture conditions. All data are representative of three independent experiments carried out using biological replicates. The error bar represents the standard error of the mean.

alignment shows no homologs outside slow growing pathogenic mycobacteria. Transmembrane region was predicted using TMMHM (http://www.cbs.dtu.dk/services/TMHMM-2.0/)

3.2. Generation of the Δ*lpqS* mutant in *M. tuberculosis*

A gene-disrupted strain of *M. tuberculosis* not expressing LpqS was constructed by phage-mediated allelic exchange (Bardarov et al. 2002). The coding region of *lpqS* was replaced by the hygromycin resistance gene and the *sacB* cassette in *M. tuberculosis* H_{37}Rv. Allelic replacement was confirmed in the mutant by PCR and Southern blotting (Fig. S1). As described in Fig. S1, the allelic exchange mutants showed a single hybridizing fragment of size 6.1 kb and for the wild-type strain the fragment was around 2.1 kb. The disrupted strain was then complemented with a copy of *lpqS* cloned under the hsp60 promoter in the vector pMV361 (integrative backbone) and the complementation confirmed by PCR (Fig. S1).

3.3. *In vitro* growth kinetics

To determine whether *lpqS* disruption has brought in any change in the *in vitro* cultivation, we compared the growth profiles of Δ*lpqS*, CΔ*lpqS* and the wild type H_{37}Rv under standard culture conditions in enriched Middlebrook 7H9 media and in Sauton's minimal media. All strains showed similar doubling times in normal 7H9 media (Fig. 1). However, the growth of the mutant in Sauton's minimal media started to decline after day 7 and reduced by more than one log after day10. The growth of the complemented strain was more compared to the mutant but not comparable to H_{37}Rv (Fig. 1).

3.4. Sensitivity to SDS

Reduced viability of the mutant in Sauton's minimal media prompted us to compare the sensitivity of wild type H_{37}Rv and the mutant strain to cell-wall active compound like SDS; and these were analyzed to determine whether the knockout of the gene has resulted in defective cell wall homeostasis in mycobacteria. CFU of the mutant was almost reduced by more than one log when grown on 7H10 plates containing 0.001% SDS and reduced by more than two logs on plates containing 0.01% SDS (Fig. 2). Selective deletion of *lpqS* resulted in interrupted cell wall homeostasis making the mutant more sensitive to cell-wall active compound like SDS. Complementation only partially restored the original phenotype comparable to *M. tuberculosis* H_{37}Rv.

3.5. Biofilm formation and colony morphology

Typical mature biofilms comparable to H_{37}Rv were formed by both the mutant and the complemented strains. Hence gene disruption has not affected the cell to cell interaction of the bacilli. No obvious difference in the colony morphology of the mutant and the complemented strain was observed when compared with the wild type H_{37}Rv (data not shown).

3.6. Sensitivity to copper

Earlier studies have identified *lpqS* to be a member of five locus copper-inducible regulon (Festa et al. 2011). Hence the sensitivity of wild type H_{37}Rv and the mutant strain to varying copper concentrations were compared. All the three strains grew normally on plain 7H10 plates. The growth of the mutant was impaired on 7H10 plates containing copper. CFUs of the Δ*lpqS* mutant decreased by one log in plates containing 25 and 50 μM copper sulphate and by more than two log in 7H10 plates containing 75 and 100 μM copper sulphate. Complementation of the mutant was not very effective (Fig. 3). Further studies carried out to assess the growth of the mutant under conditions unrelated to copper showed that mutant exhibited normal growth characteristics like wild type H_{37}Rv when grown on solid media containing 100 μM of zinc and 0.05% iron though it exhibited growth reduction in the presence of 100 μM of copper (Fig. 4).

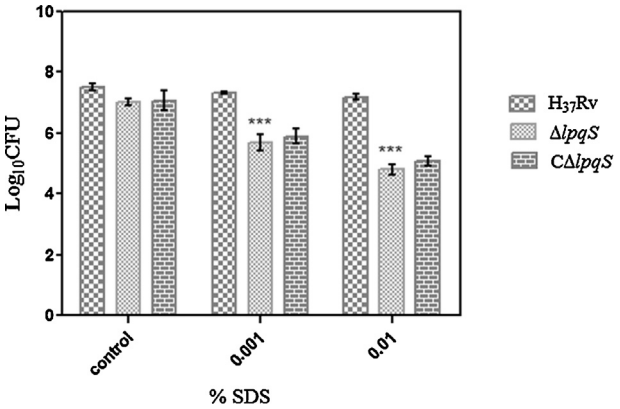

Fig. 2. Sensitivity of *M. tuberculosis* strains to SDS. Ten-fold serial dilution of logarithmic phase mycobacterial cultures (10 ml) were spotted on 7H10-ADS agar medium containing no SDS, 0.001% SDS and 0.01% SDS and incubated at 37 °C. Growth was recorded 4–6 weeks after incubation.

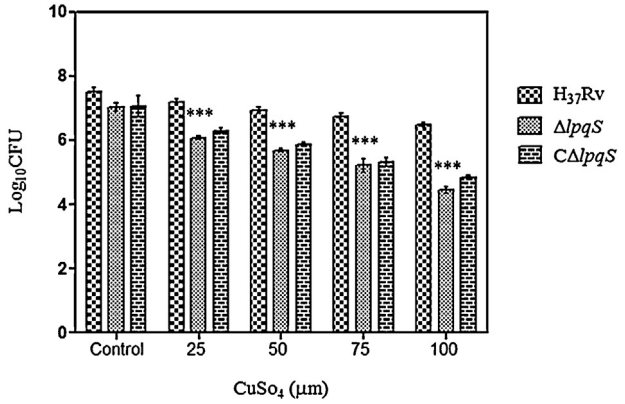

Fig. 3. Sensitivity of *M. tuberculosis* strains to copper. Ten-fold serial dilution of the logrithmic phase mycobacterial cultures (10 ml) were spotted on 7H10-ADS plates containing no copper and plates containing 25, 50, 75 and 100 μM copper and incubated at 37 °C. Growth was recorded 4 weeks after incubation.

3.7. Intracellular survival of ΔlpqS mutant in THP1-differentiated macrophages

The intracellular viability of H_{37}Rv, Δ*lpqS* and CΔ*lpqS* was assessed by infecting the THP1 differentiated macrophages. Viability counts on day 0 showed no difference among the strains. This clearly shows that the mutant and the complemented strains were not defective in infectivity. However viability counts made at later time points revealed that the mutant strain was attenuated for growth in macrophages compared to wild type H_{37}Rv (Fig. 5). Complementation of the mutant only partially restored this growth defect of the mutant observed within macrophages.

3.8. Cytokine ELISA

Cell-free culture supernatants of the infected cell lines were analyzed for the levels of TNF-α, IL-12 and IL-6 by sandwich ELISA using BD optEIA ELISA kit (BD pharmingen, USA) as per the manufacturers protocol. Significant reduction in the levels of cytokines TNF-α, IL-12 and IL-6 was observed with mutant-infected THP-1 derived macrophages (Fig. 6). Increased level of IL-12 was observed in macrophages infected with the complemented strain compared to wild type H_{37}Rv.

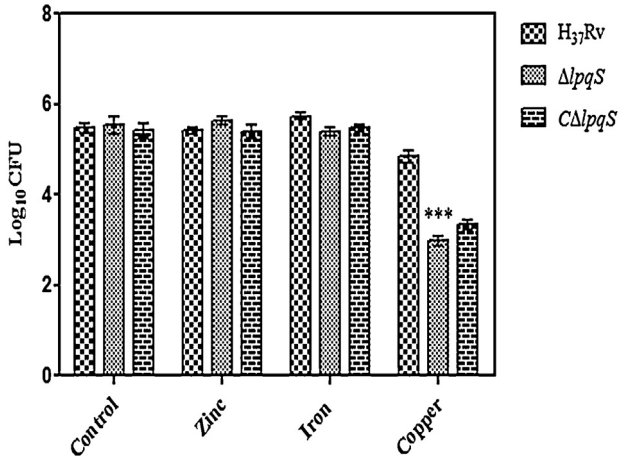

Fig. 4. Sensitivity of *M. tuberculosis* strains to copper, zinc and iron. Ten-fold serial dilutions of the logrithmic phase mycobacterial cultures (10 ml) were spotted on plain 7H10-ADS plates and plates containing 100 μM of zinc sulphate, 100 μM of copper sulphate and 0.05% Ferric ammonium citrate and incubated at 37 °C. Growth was recorded 4 weeks after incubation.

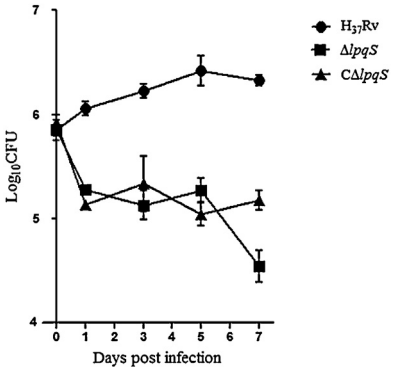

Fig. 5. Intracellular growth of Δ*lpqS* mutant within THP1 derived macrophages. THP-1 derived macrophages were infected with wild type H_{37}Rv, Δ*lpqS* and CΔ*lpqS* for 4 h. Infected cells were then lysed with ice-cold 1% trypsin in RPMI and plated for counting CFUs after making serial dilutions at specified time points. All data are representative of three independent experiments carried out using biological replicates. The error bar represents the standard error of the mean.

3.9. RT-PCR analysis

Unsuccessful attempts to achieve full complementation of the mutant with a single copy of the *lpqS* gene cloned under the hsp60 promoter prompted us to look for the possibility that *lpqS* forms an operon with the genes downstream. RTPCR analysis was used to identify whether *lpqS* and the three genes downstream are cotranscribed using primers amplifying intergenic regions of the gene cluster. cDNAs were synthesized from RNAs of *M. tuberculosis* using Quantitect Reverse transcriptase kit from Qiagen. Transcripts were detected for all intergenic junctions tested. Negative control experiment were performed by excluding the Reverse transcriptase enzyme during RT reactions, which also served as means of confirming the absence of DNA contamination in RNA samples (Fig. 7)

4. Discussion

Attenuated phenotype of the lipoprotein mutants and mutants lacking genes of its biosynthetic pathway clearly demonstrates the importance of lipoproteins in the virulence of *M. tuberculosis* (Sulzenbacher et al. 2006; Tschumi et al. 2009). Intracellular pathogens like *M. tuberculosis* requires adaptation to varying environmental conditions prevailing inside the host cells like hypoxia for long term survival. LpqS is a putative lipoprotein of *M. tuberculosis* implicated in its survival under hypoxic conditions. (Muttucumaru et al. 2004; Gonzalo-Asensio et al. 2008). In addition recent studies have identified *lpqS* as a member of the five locus copper inducible regulon (Festa et al. 2011). Thus we hypothesized that *lpqS* might be required for the survival of the pathogenic mycobacteria under specific niche inside the host.

In the present study we generated a gene knockout mutant of *lpqS* in *M. tuberculosis*. Successful deletion of *lpqS* from the genome of *M. tuberculosis* indicates that the gene is not essential for *M .tuberculosis*, thereby confirming the earlier reports (Sassetti et al. 2003). We then compared the growth characteristics of the mutant under different *in vitro* growth conditions. The mutant showed growth characteristics similar to H_{37}Rv in enriched Middlebrook 7H9 media but grew slower than the wild type in nutrient deprived Sauton's minimal media. Furthermore, the mutant was found to be more susceptible to SDS when grown on solid media. This differential growth characteristics of the mutant and its increased susceptibility to cell-wall active compounds like SDS shows that deletion of *lpqS* has altered the cell-wall homeostasis. Interupted cell-wall homeostasis often leads to such collateral phenotype.

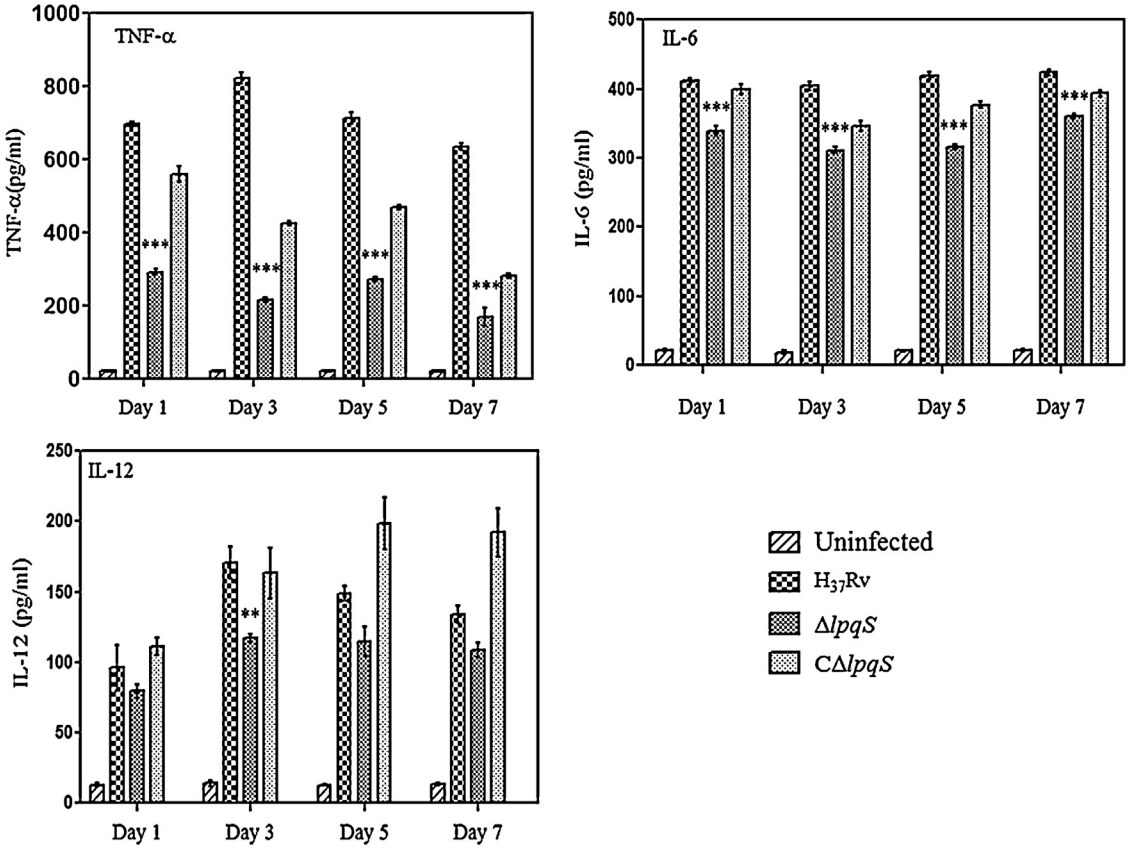

Fig. 6. Enzyme linked immunosorbent assay for TNF-α, 1L-12 and IL-6. Levels of cytokines secreted by macrophages infected with wild type ($H_{37}Rv$), Δ*lpqS* mutant and complemented strains CΔ*lpqS* were measured by sandwitch ELISA using manufacturer's protocol. The capture antibody was coated on the ELISA plate.The samples were then added, followed by the detection antibody.Optical density was then read by using spectrophotometer after the addition of the substrate. Figure shows the TNF-α, 1L-12 and IL-6 levels at different time points of infection.

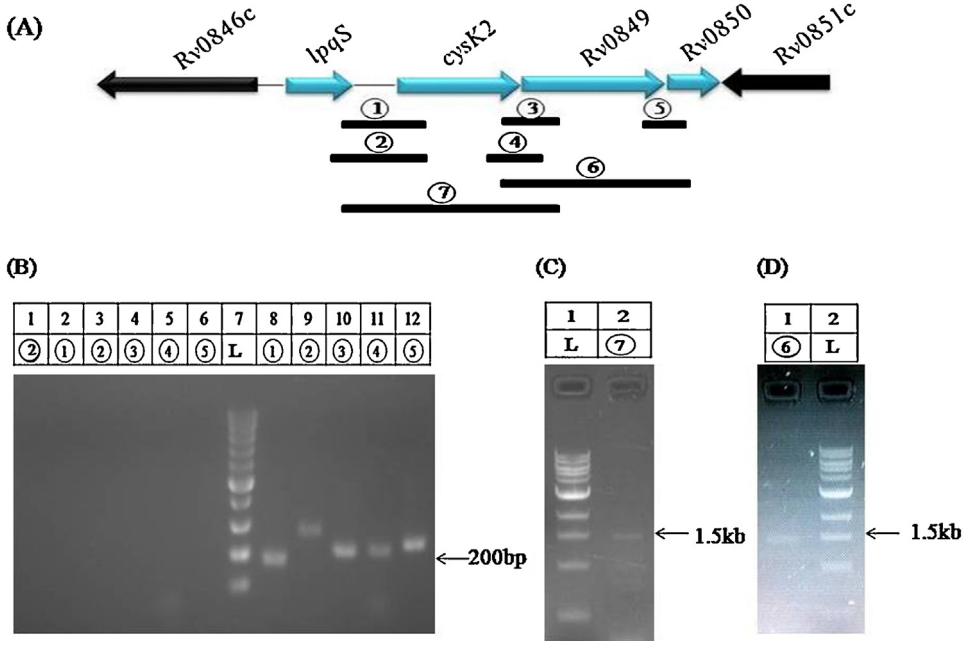

Fig. 7. RT-PCR to establish cotranscription of *lpqS*, *cysK2*, Rv0849 and Rv0850 Total RNA from copper induced *M. tuberculosis* culture was analyzed by RT-PCR. Suitable primers were used to amplify the intergenic regions between the gene cluster *lpqS*-Rv0850 using cDNA as template. (A) Blue arrows represent the genes of the *lpqS* operon. Black bars represents the PCR amplified intergenic regions of the *lpqS* operon using cDNA synthesized from *M. tuberculosis* total RNA. Black bars are numbered on top to denote the intergenic regions amplified by RT-PCR. Primer pairs used to amplify the intergenic regions are listed in Table 3. (B) RT-PCR analysis of the intergenic regions of the *lpqS*-Rv0850 operon.Top row represents the lane numbers and numbers in the bottom row represents the corresponding intergenic region. Lane 1: PCR amplification of intergenic region 2 using RNA as template. Lane 2–6: RT −ve controls (Reverse transcriptase enzyme omitted during RT Reactions) for intergenic regions 1–5; Lane 7 (L): 100 bp ladderLane 8–12: Transcripts for intergenic regions numbered 1–5 using cDNA from *M. tuberculosis* (C) Lane 1: 1 kb ladder; Lane 2: Transcript for the region denoted by black bar 7; (D) Lane 1: Transcript for the region denoted by black bar 6; Lane 2: 1 kb ladder.

Transcriptome profiling of *M. tuberculosis* has identified the upregulation of *lpqS* and the genes downstream under conditions like hypoxia and increased copper concentrations (Festa et al. 2011; Muttucumaru et al. 2004). In support of these findings our experiments with copper and other biologically relative metals like zinc and iron found *lpqS* mutant to be more susceptible to killing by higher levels of copper but not to other metal ions tested when grown on solid media. These increasing evidences suggests that *lpqS* has a role in copper related response of *M. tuberculosis*.

Macrophages are routinely used to study the virulence of mycobacterial strains. Severely impaired replication of the mutant inside macrophages strongly suggests that *lpqS* is required for the intracellular survival of *M. tuberculosis*. Hypoxic conditions prevailing inside mycobacterial granulomas are known to induce copper uptake inside macrophages through macrophage copper transporter CTR1 (White et al. 2009). Besides this, the copper levels were found to be elevated in cultured macrophages infected with *M. tuberculosis* (Wagner et al. 2005). Thus in response to such increased copper levels *in vivo*, *M. tuberculosis* might put on copper-inducible regulons like *lpqS*. However, the precise role of such copper inducible regulons in the pathogenesis of *M. tuberculosis* still remains unknown.

Macrophages infected with the mutant strain secreted lower levels of cytokines TNF-α, IL-6 and IL-12 than the macrophages infected with wild type. Reduced survival of the mutant inside macrophages correlates well with the concurrent reduction in the levels of pathogen driven cytokines. However, the macrophages infected with the complemented strain secreted increased levels of IL-12 and was found to be significant compared to H_{37}Rv. Microbacterial lipoproteins are potent stimulators of IL-12 production which was found to be independent of TNF-α secretion in macrophages (Brightbill et al. 1999). 19 kDa lipoprotein was reported to induce IL-12 secretion when its concentration increases above the physiological level encountered during wild type infection (Stewart et al. 2005). Thus, the significant increase in the secretion of IL-12 cytokine by the macrophages infected with the complemented strain might be attributed to the increased levels of LpqS expression under the hsp60 promoter when compared with its expression under tightly regulated copper-inducible promoter in the wild type. Decreased intracellular survival and increased IL-12 response exhibited by the complemented strain in macrophages are both associated with protective immune response and could open new avenues for vaccine development.

Lack of full complementation observed when the mutant was transformed with a copy of plasmid (integrative backbone) bearing *lpqS* under the strong hsp60 promoter prompted us to look for the possibility of *lpqS* to be cotranscribed as a single operon with the genes downstream. Here, we demonstrate through RT-PCR experiments that *M. tuberculosis lpqS* is the first gene of the transcriptional unit that contains the ORFs cysK2, Rv0849 which encodes an S-type transporter and Rv0850 and this operon is restricted to slow growing pathogenic mycobacteria. Hence further studies with the complemented strains carrying all the four genes of the operon would be necessary to attain complete complementation of the mutant.

To summarize, *lpqS* and other genes of the copper-inducible RicR regulated regulon may play essential role in *M. tuberculosis* replication and survival inside the host cell. Hyper-resistance of *M. tuberculosis* RicR mutant to increased copper levels *in vitro* indicate that these copper-inducible regulons may function to protect *M. tuberculosis* against copper toxicity. Alternatively, copper ions may serve as a signal to induce the expression of these regulons in *M. tuberculosis* that may in turn enable the survival of *M. tuberculosis* inside the host under specific niche. The conservation of these genes across diverse Mycobacterium species further ensures preservation of common functionality. Further studies are necessary to address these issues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2013.02.007.

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