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MOLECULAR ASPECTS

Towards understanding the biological function of the unusual chaperonin Cpn60.1 (GroEL1) of *Mycobacterium tuberculosis*

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SUMMARY

The 60 kDa heat shock proteins, also known as Cpn60s (GroELs) are components of the essential protein folding machinery of the cell, but are also dominant antigens in many infectious diseases. Although generally essential for cellular survival, in some organisms such as *Mycobacterium tuberculosis*, one or more paralogous Cpn60s are known to be dispensable. In *M. tuberculosis*, Cpn60.2 (GroEL2) is essential for cell survival, but the biological role of the non-essential Cpn60.1 (GroEL1) is still elusive. To understand the relevance of Cpn60.1 (GroEL1) in *M. tuberculosis* physiology, detailed transcriptomic analyses for the wild type H37Rv and *cpn60.1* knockout (*groEL1-KO*) were performed under *in vitro* stress conditions: stationary phase, cold shock, low aeration, mild cold shock and low pH. Additionally, the survival of the *groEL1-KO* was assessed in macrophages at multiplicity of infection (MOI) of 1:1 and 1:5. We observed that survival under low aeration was significantly compromised in the *groEL1-KO*. Further, the gene expression analyses under low aeration showed change in expression of several key virulence factors like two component system PhoP/R and MprA/B, sigma factors SigM and C and adversely affected known hypoxia response regulators Rv0081, Rv0023 and DosR. Our work is therefore suggestive of an important role of Cpn60.1 (GroEL1) for survival under low aeration by affecting the expression of genes known for hypoxia response.

1. Introduction

Pathogenic bacteria face drastic environmental changes on being phagocytosed by host cells, including: oxidative burst, low pH and limited supply of O_2 and nutrients [1–4]. Many pathogens adjust to these changes by increasing the production of "heat shock proteins (Hsp's)" or chaperones. The importance of heat shock proteins for survival in the host can be gauged by monitoring mutants that either over-express or lack Hsps. For example, in *Listeria monocytogenes*, while increased expression of ClpC, a Clp family heat shock protein has been shown to release bacteria from the phagosomal compartment to the host cytosol accompanied by rapid rounds of multiplication; deletion of *clpC* leads to an accumulation of bacteria in the phagosomal compartment with reduced bacterial load in the target organs [5,6]. Hsps are also major antigens in infectious diseases caused by bacteria such as *Chlamydia trachomatis, Borrelia burgdorferi, Bordetella pertussis, Helicobacter pylori, Mycobacterium leprae, Mycobacterium tuberculosis* etc. [7–9]. Thus, Hsps are potential virulence factors in several pathogenic bacteria.

M. tuberculosis (Mtb) remains a major global health hazard killing 1.4 million people every year [10]. A detailed understanding of the disease causing organism and its virulence factors is a prerequisite for unravelling its modes of pathogenesis. One of the earliest antigens of Mtb identified, Heat shock protein 65 (Hsp65), belongs to the GroEL or the chaperonin 60 (Cpn60) family of

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proteins [11,12]. Interestingly, while the *Escherichia coli* genome possesses only one copy of the *cpn60* gene, Mtb has two paralogs of this gene. The current nomenclature for these chaperonin proteins is Cpn60.1 or GroEL1 for Hsp60, and Cpn60.2 or GroEL2 for Hsp65. These two genes are coded separately on the Mtb genome; *groEL1* (Rv3417c) in an operonic arrangement with *groES* (Rv3418c) and *groEL2* (Rv0440) separately on the chromosome. Although, both the GroEL proteins are up regulated during heat shock [13], oxidative stress response [14], macrophage infection [15] and are implicated in the immune response to Mtb infection [16–18], only *groEL2* and *groES* are essential genes, whereas *groEL1* is dispensable [19]. *groEL1* knockouts in other mycobacterial strains, *Mycobacterium smegmatis* and *Mycobacterium bovis BCG* are also viable [20,21], although the *M. bovis BCG groEL1* knockout shows increased sensitivity to oxidative stress (H_2O_2) [21]. A role for GroEL1 in maintaining cell wall integrity is suggested by it interacting with the mycolic acid pathway protein, KasA [20]. However, the functional role of GroEL1 in phylogenetically related mycobacteria still remains enigmatic.

Previous studies in our laboratory have shown that though GroEL1 and GroEL2 share high sequence similarity with *E. coli* GroEL, they are uncanonical chaperonins that form dimers even in the presence of ATP and GroES [22], and display weak ATPase activity as against trademark tetradecameric *E. coli* GroEL [22]. Although Mtb GroEL1 can complement for the absence of *M. smegmatis groEL1* and Mtb GroEL2 can weakly complement for the absence of *M. smegmatis groEL2* gene [23], contradictory results were reported on the ability of the Mtb GroELs in complementing *E. coli groEL* mutant [23,24]. Studies by other groups have highlighted a role of Mtb GroELs in activating an immune response, wherein GroEL2 acts as a major adhesion factor for binding to monocytes [25] and GroEL1 is a potent inducer of human monocyte cytokines [26,27]. Thus, these two proteins could each have more than one distinct function [28].

Another observation from our laboratory had earlier revealed that GroEL1 binds to DNA with high affinity, thus behaving like a nucleoid associated protein [29]. To decipher the biological function of GroEL1, we studied here the gene expression profiles of Mtb *H37Rv* (WT) and Mtb *groEL1* knockout (*groEL1-KO*) under various stress conditions by microarray. We compared microarray profiles of WT and *groEL1-KO* in stationary phase, cold shock, low aeration, mild cold shock and low pH. We simultaneously studied the survival and growth of WT and *groEL1-KO* under these *in-vitro* stress conditions and during macrophage infections. We report results of these studies, and propose a functional role for GroEL1 in adaptation to low aeration. It however remains unclear whether this function of GroEL1 is due to its chaperoning ability or its DNA binding property. We therefore believe that post-gene duplication, GroEL1 might have evolved the specialized function of adaptation to low aeration, whereas GroEL2 might have retained the function of a generalized chaperonin.

2. Materials and methods

2.1. Strains and culture conditions

The Mtb H37Rv (WT), *groEL1* knockout (*groEL1-KO*) and complement (*groEL1*-Comp) strains were a kind gift from Dr Anil Ojha. They had constructed the *groEL1-KO* and *groEL1*-Comp as follows: For constructing *groEL1-KO*, a ~500 bp upstream and ~100 bp downstream of the start site of the ORF were cloned into AflII-XbaI sites of pYUB854. Similarly, ~100 bp upstream and ~500 bp downstream of the stop site were cloned in NheI-BglII sites of the pYUB854. The recombinant plasmid was packaged in TM4 derivative phAE87 and the phage was delivered into Mtb through transduction. Hygromycin resistant colonies were selected and screened for gene replacement by Southern blot. The clone containing a replacement of *groEL1* gene with Hygromycin cassette was chosen for further studies. For generating *groEL1*-Comp, L5-based integrative plasmid pMH94 was used as vector to clone Mtb *groEL1* along with its 500 bp upstream region. The *groEL1*-Comp was selected for Kanamycin (Kan). The absence of GroEL1 in *groEL1-KO* was confirmed by Western using GroEL1 monoclonal antibody [29] (Supplementary Figure 1). For microarray experiments, WT and the *groEL1-KO* were grown at 37 °C in Middlebrook 7H9 supplemented with ADC and 0.05% Tween (Beckton Dickinson) in rolling culture. Additionally, 50 μg/ml of Hygromycin (Hyg) was added to the *groEL1-KO* culture. Working stocks were expanded from frozen aliquots shortly before the experiments began. Briefly, 10 ml culture was grown to mid log phase ($OD_{600nm} = 0.6$) before subjecting to the specified stress conditions. For stress conditions, i.e. low pH, mild cold shock and cold shock, the cultures were pelleted at 2000 g for 5 min after 2 h of stress, frozen on dry ice and stored at −80 °C until processed for RNA. For low aeration and stationary phase, the same was done after the condition was reached.

2.2. In vitro growth under stress conditions

A series of 10 ml cultures were used to determine colony forming units (CFU) counts after exposure to various stress conditions. For cold shock, the cultures were incubated on ice for 2 h; for low aeration, the cultures were kept standing in 37 °C incubator for 24 h; for low pH, 7H9 media at pH 5.5 was used. CFU plating was done after 2 h for low pH, cold shock and after 24hr for low aeration on 7H11 agar plates for WT, 7H11 agar with 50 μg/ml Hyg for *groEL1-KO* and 7H11 agar with 50 μg/ml Hyg and 30 μg/ml Kan for the *groEL1*-Comp strain. For stationary phase cultures, the cultures were grown till they reached an OD_{600nm} of greater than 2.0. For extended low aeration stress, 10 ml of cultures were overlaid with 2 ml of paraffin oil (in 50 ml tubes) and kept standing for a period of 7 days. At the end of day 1 and day 7, the layer of oil was removed, cells vortexed and plated for CFU. At each time point there were at least three biological replicates. Results were expressed as percentage of bacteria surviving after stress to total number of bacteria present before being subjected to stress condition.

2.3. RNA extraction, labelling of probes and microarray analysis

The cell pellets were resuspended in Trizol, transferred to a tube containing Lysine Matrix B (QBiogene, Inc.) and vigorously shaken at a maximum speed for 30sec in FastPrep 120 homogenizer (QBiogene) three times, with cooling on ice between these steps. The subsequent steps were performed as per Rustad et al. [30]. 3 μg of total DNAse treated RNA was used to prepare cDNA labelled using aminoallyldUTP (Fermentas). Fluorescent Cy3 and Cy5 dyes (Amersham) were then attached to the aminoallyl tags. Each pair of differentially labelled probes was resuspended in 60 μl of hybridization buffer (500 ml formamide, 250 ml 20X SSC, 5 ml 10% SDS, 245 ml ultrapure water) and hybridized to 12 × 135 K microarrays from Roche–Nimblegen overnight in a 42 °C incubator. Slides were then washed in increasingly stringent wash conditions (5 min 1X SSC 0.1% SDS, 10 min 0.1X SSC 0.1% SDS, 4 times 1 min in 0.1 X SSC and a final 10 s wash in 0.01X SSC). Arrays were scanned and spots were quantified using Genepix 4000B with GenePix 6.0 software. This data was then normalized using ArrayStar Software and data exported to excel sheet. Significance was defined as having a 3-fold change and false discovery rate (FDR) of less than 0.4% was calculated using SAM in R package for a minimum of three arrays. Fully annotated microarray data has been deposited in GEO repository (Accession number GSE68856).

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2.4. Quantitative real time PCR

Real time PCR was performed for a set of 7 genes specific for low aeration condition for WT, *groEL1-KO* and *groEL1*-Comp strain. RNA was harvested as mentioned above. First strand cDNA synthesis was performed using 500 ng of RNA with iScript Select cDNA Synthesis Kit (Bio-Rad) using random oligonucleotides. PCR was performed using gene specific primers listed in Supplementary Table 1. Expression of genes was analysed with real-time PCR using iQ SYBR Green Supermix (Bio-Rad) and a BioRad iCycler. For comparisons between the expression ratios in WT, *groEL1-KO* and *groEL1*-Comp, the induction ratio for each gene was normalized to *sigA* gene expression.

2.5. Infection of THP1 cell line

THP-1 cells were cultured in RPMI-1640 and supplemented with 5% heat inactivated FCS, 2 mM glutamine, penicillin (100i.u/ml) and streptomycin (100 μg/ml) at 37 °C in 5% CO_2 humidified incubator. THP1 cell line in passages 3–5 were used for the assay. The cells which were grown to a density of $2–10 \times 10^5$ cells per ml at 37 °C in a humidified, 5% CO_2 atmosphere, were differentiated with 30 ng/ml phorbol 13-myristate 12–acetate (PMA) for 48 h. Non adherent cells were removed by washing twice in warm RPMI 1640 and the resulting monolayers were covered with supplemented RPMI growth media. Mid exponential cultures of WT, *groEL1- KO* and *groEL1*-Comp strains were centrifuged at 5000 g for 10 min and resuspended in antibiotic free RPMI 1640 growth media. Single cell suspensions were made by serial passing of these cultures through 23, 26 and 30 gauge needle. These were then added to the macrophage monolayer at an infection ratio of either 1 bacterium per macrophage or 5 bacilli per macrophage. The plates were then centrifuged at 1000 g for 3 min. The plates were incubated for 4 h at 37 °C in a humidified 5% CO_2 incubator. The residual bacteria were removed by decanting the supernatant and adding RPMI1640 supplemented with 0.6 μg/ml amikacin. After 2 h of amikacin treatment, the cells were resuspended in warm supplemented RPMI media. CFU plating was done at 6 h, 24 h, 48 h and 72 h after infection.

2.6. Transcriptional network analysis (TRN analysis)

To determine the transcription factors whose activity under stress conditions is affected in the *groEL1-KO*, we first compiled a transcriptional regulatory network for Mtb by merging three published resources: a curated network from Sanz et al. [31] comprising 83 TFs and 3212 regulatory links; another curated regulatory network from Rohde et al. [32] which is composed of 1801 regulatory associations spanning 83 TFs and the third network, which was obtained from transcription factor (TF) over expression data published in Rustad et al. [30], from which effective 'target' genes were identified for each of the 183 TFs by applying a threshold of FDR < 0.01 and 2-fold expression changes. The resulting non-redundant network describes 12,444 regulatory interactions spanning 206 TFs and a total of 3310 genes.

For all the five stress conditions studied, *groEL1*-KO vs. WT expression data was mapped on this network and for each TF, statistical significance of the association of its target set with the subset of differentially expressed genes was evaluated by applying Fisher's exact test. TFs with a Bonferroni-corrected p-value < 0.05 were identified as being differentially active between WT and *groEL1-KO* stress response. TFs involved in the WT stress response were separately determined by applying the above procedure to the expression data for WT stress vs. WT log comparison.

3. Results

3.1. Gene expression profiling of WT and groEL1-KO

Gene expression profiling was performed at mid log phase ($OD_{600nm} = 0.6$) as well as for the five stress conditions (Materials and Methods). Gene expression analysis of *groEL1-KO* in log phase vs. WT in log phase did not reveal any genes crossing the cut-off of 3-fold change (FDR < 0.4% or at a higher FDR of 10%). This suggests that the *groEL1-KO* has minimal effect on the gene expression pattern under normal growth conditions. This observation is consistent with the growth curves of WT and *groEL1-KO*, where no effect of *groEL1* deletion was found on the growth rate of the cultures (Supplementary Figure 2).

A comparison of gene expression profiles of *groEL1-KO* vs. WT in the five stress conditions revealed a large number of genes being differentially expressed. At a 3-fold cut off and FDR < 0.4%, the number of differentially expressed genes ranged from 17 (in low pH) to 394 (Stationary Phase) (Supplementary Table 2; Supplementary text). As GroELs belong to the family of heat-shock proteins, we checked the expression of various heat-shock proteins in the WT strain grown under various stress conditions and compared the relative differences in their expression levels with respect to that in the log phase (Supplementary Table 3). Here, the gene expression in WT strain grown to log phase was taken as control to present the relative differences in expressions of heat-shock proteins. Among the chaperonin genes, we observed substantial up regulation in expression of *groEL2* (15-fold, 11-fold) and *groES* (10-fold, 6-fold) in WT under cold shock and low aeration respectively. It was also worthwhile to note a 14-fold upregulation of *groEL1* under low aeration (Supplementary Table 3). To study the consequence of the drastic differences in the expression of GroEL1 under different stress conditions, we compared the survival of WT, *groEL1-KO* and *groEL1*-Comp strain under cold shock, low aeration and stationary phase. Under all these conditions, except low aeration, the growth rates of the WT, *groEL1-KO* and *groEL1*-Comp were not significantly different from each other (Supplementary Figure 3). Under low aeration for standing cultures, the *groEL1-KO* was observed to survive 40% less than the WT in 24 h (Figure 1A). Furthermore, the growth of *groEL1-KO* was significantly compromised in more stringent low aeration conditions, i.e. by addition of paraffin oil over the standing cultures, on Day 1 and Day 7 as compared to the WT (Figure 1B). Although the biological replicates exhibited variation, the percentage survival increased upon GroEL1 complementation (Figure 1A and B). With no differences in the growth of *groEL1-KO* with WT in any other stress condition, we checked if the *in-vivo* survival of *groEL1-KO* is compromised as compared to WT. No significant differences in the CFU counts for WT and *groEL1-KO* at 4 time points taken after infection in THP1 cell line were observed (Supplementary Figures 4A and B). With these observations, we could conclude that the absence of GroEL1 affects the ability of Mtb to survive under low aeration, with no significant effect under other stress conditions tested.

3.1.1. Differential gene expression under low aeration stress

Though an exhaustive transcriptomics was performed spanning five different stress conditions to assess the physiological role of GroEL1 in Mtb, with the observation that GroEL1 helped Mtb survival in low aeration stress, we restrict the discussion here to the differential gene expression of *groEL1-KO* under low aeration with respect to WT under low aeration. The complete dataset including all stress conditions is available in GEO repository (Accession number GSE68856).

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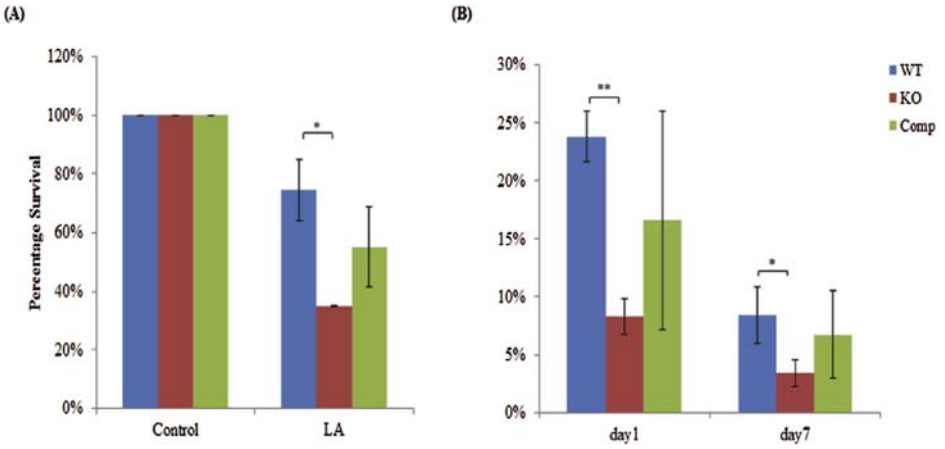

Figure 1. Survival plot of WT, *groEL1 − KO* and *groEL1*-Comp strains under low aeration stress (LA). (A) To simulate low aeration, log phase cultures were incubated for 24 h at 37 °C without shaking. Plating for CFU was done before and after application of the stress condition. These results represent mean with standard deviation from three biological replicates. (B) Percentage survival of WT, *groEL1-KO* and *groEL1*-Comp strain under low aeration stress as in (A) but with 2 ml of paraffin oil added on the standing cultures. At the end of Day 1 and Day 7, the cells were plated for CFU. These results represent mean and standard deviation from three biological replicates. Line above the bars indicates the groups that were compared to calculate the p-value (using one way Anova in SigmaPlot). * represents $p < 0.05$, ** represents $p < 0.001$.

The influence of oxygen shortage on Mtb is characterized by declining metabolic activity [33], increase in expression of genes involved in anaerobic respiration, reductive phase of tricarboxylic acid (TCA) cycle [34–36] and up regulation of lipid metabolism genes [37–39]. A total of 68 genes were differentially expressed in the *groEL1- KO* under low aeration as compared to the WT under low aeration at a 3-fold cut off (FDR < 0.4%). At three-fold cut off, 27 genes were down regulated and 41 up regulated (Table 1 lists these genes). Microarray results were confirmed by real time PCR (RT-PCR) for a set of 7 genes for all three strains WT, *groEL1-KO* and *groEL1*-Comp under low aeration condition. Out of 7, 4 genes (*Rv0848 − 0850, Rv2450c*) were down regulated in *groEL1-KO* and 3 (*Rv1168-1169, Rv0166*) were up regulated with respect to WT. These genes followed the same pattern as observed in the array (Figure 2).

An interesting observation pertained to the copper responsive genes, which are 30 in number in the Mtb genome [40]. Nine of these genes were down regulated under low aeration in the *groEL1-KO,* suggesting an importance of copper response with a high significance ($p < 10^{-9}$). Expressions of copper responsive regulators - RicR (Regulated in copper Repressor, Rv0190) [41] and CsoR (Copper sensitive operon Repressor, Rv0967) [42] were decreased by log fold change values of −1 and −1.54, respectively in the *groEL1-KO*. In addition, a decrease in the expression of the RicR regulated genes *Rv0847-Rv0850* (~0.10 log fold change) and *Rv2963* (0.11 log fold change) [41,43] was also observed in the *groEL1-KO*. Further, a decrease in the expression of CsoR target genes *Rv0968 − Rv0970* (~0.20 log fold change) was observed in the *groEL1-KO*. *Rv0847, Rv0968* and *Rv2963* encode for membrane proteins and *Rv0969* encodes for a copper exporter CtpV [44]. Displacement of Fe–S clusters by increased copper toxicity is known to stimulate synthesis of hydratases, *leuCD*, of branched amino acid synthesis pathway [45]. We too observed a 3-fold increase in the expression of orthologs in Mtb genes *Rv2987c-88c* (*leuCD*) in the *groEL1-KO* under low aeration relative to the WT. Thus, it may be hypothesized that the decreased levels of membrane proteins and copper exporter protein lead to an increased accumulation of copper in cytoplasm, possibly adversely affecting growth of *groEL1-KO*. It is highly tempting to speculate that the His-rich C-terminal tail of GroEL1 protein might be involved in copper sequestration, absence of which leads to copper accumulation in the cytoplasm of the *groEL1-KO*.

Comparison of the WT and *groEL1-KO* array data under low aeration stress revealed decrease in expression of *icl* (0.33 log fold change) and *purU* (0.26 log fold change). Isocitrate lyase (ICL) is one of the major enzymes in adaptation to limited availability of oxygen [46,47]. It helps in adaptation in a dual manner: as a gate enzyme for glyoxylate shunt pathway and additionally by preventing accumulation of toxic propionyl CoA [48]. *purU* encodes for formyl tetrahydrofolate deformylase. Along with *purU,* a decrease in the expression was also observed for genes in folate synthesis super pathway [49,50] (*Rv3606c-3609c*), *Rv3356c* (*folD*) and *Rv2447c* (*folC*) (log fold change ranging from −1.58 to −1.0). Folate is an important cofactor in several biochemical reactions including purine and pyrimidine synthesis, amino acid synthesis and pyruvate dehydrogenase complex [51,52].

Exit from dormancy is characterized by reestablishment of metabolic and replicative activity, initiated by the secreted proteins known as resuscitation promoting factors (Rpf) [53]. Mtb possesses 5 Rpf homologues: Rpf A-E [54]. On comparison of expression profiles of *groEL1-KO* vs. WT under low aeration condition, a decrease in expression for *Rv0867c* (*rpfA*) (0.33 log fold change) and *Rv2450c* (*rpfE*) (0.05 log fold change) was observed. The importance of resuscitation promoting factor RpfE is highlighted by its essentiality for transition from a slow to fast growth in culture [55], its role under hypoxia [56] and as a potential vaccine candidate because of its ability to simultaneously induce both Th1- and Th17-polarized T-cell expansion [57]. We therefore hypothesize that one of the several possibilities explaining the decrease in survival observed under low aeration could be due to the decrease in the expression of genes involved in basic cell metabolism.

3.1.1.1. Transcriptional regulatory network response in groEL1-KO. To understand how the *groEL1-KO* adapts to various stresses, we overlaid microarray data onto a transcriptional regulatory network as described in Materials and Methods [30–32] and arrived at a set of 68 significant ($p < 0.05$) sub-networks/regulons that were affected either in the WT or the *groEL1-KO* on being subjected to stress conditions (Supplementary Figure 5). To begin with, we compared the regulons of WT grown to log phase in normal growth conditions and WT subjected to low aeration stress (WT LA). Low aeration stress affected 18 regulons in WT. Seven of these regulons were also affected in the *groEL1-KO* under low aeration (KO LA)

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Table 1
Genes with *groEL1*-dependent differential expression under low aeration stress: List of genes that exhibit induction or repression in *groEL1-KO* as per the microarray data. All genes have greater than 3-fold induction or repression in the *groEL1 − KO* as compared to the WT with a FDR < 0.04%.

Gene ID	Functional categories	Product	Log fold change
	Cell wall and cell processes		
Rv0655		Ribonucleotide-transport ATP-binding protein ABC transporter mkl	3.22
Rv0841		Conserved membrane protein	3.10
Rv2620c		Conserved membrane protein	5.07
Rv2875		Major secreted immunogenic protein mpt70	3.09
Rv3289c		Transmembrane protein	3.37
Rv3479		Transmembrane protein	3.20
Rv0341		Isoniazid inductible gene protein iniB	0.29
Rv0847		Lipoprotein lpqS	0.12
Rv0849		Conserved membrane transport protein	0.10
Rv0867c		Resuscitation-promoting factor rpfA	0.33
Rv0969		Metal cation transporting P-type ATPase ctpV	0.23
Rv2450c		Resuscitation-promoting factor rpfE	0.05
Rv2963		Membrane protein	0.11
	Conserved hypotheticals		
Rv0839		Conserved hypothetical protein	3.03
Rv1682		Conserved hypothetical protein	3.56
Rv1870c		Conserved hypothetical protein	3.9
Rv2516c		Hypothetical protein	3.58
Rv2517c		Hypothetical protein	3.44
Rv2662		Hypothetical protein	4.33
Rv2817c		Conserved hypothetical protein	3.58
Rv3463		Conserved hypothetical protein	3.39
Rv0968		Conserved hypothetical protein	0.29
Rv0976c		Conserved hypothetical protein	0.24
	Information pathway		
Rv3287c		Anti-sigma factor rsbW	3.11
Rv3012c		glutamyl-tRNA(gln) amidotransferase subunit C gatC	0.27
	Insertion sequences and phages		
Rv1586c		phiRv1 integrase	4.09
Rv1587c		13E12 repeat family protein	3.65
Rv0850		transposase	0.12
	Intermediary metabolism and respiration		
Rv0711		arylsulfataseatsA	3.14
Rv0768		Aldehyde dehydrogenase NAD-dependent aldA	3.02
Rv2485c		carboxylesterase lipQ	4.01
Rv2622		methyltransferase/methylase	7.69
Rv2913c		D-amino acid aminohydrolase	7.27
Rv2988c		3-isopropylmalate dehydratase large subunit leuC	3.25
Rv3230c		oxidoreductase	3.05
Rv3251c		rubredoxin rubA	3.99
Rv0467		isocitratelyase icl	0.33
Rv0846c		Oxidase	0.23
Rv0848		Cysteine synthase A cysK2	0.09
Rv2962c		glycosyltransferase	0.30
Rv2964		formyltetrahydrofolatedeformylase purU	0.26
	Lipid metabolism		
Rv0166		Fatty-acid-CoA ligase fadD5	6.61
Rv1549		Fatty-acid-CoA ligase fadD11_1	3.19
Rv3061c		acyl-CoA dehydrogenase fadE22	3.29
Rv3229c		linoleoyl-CoA desaturase, desA3	8.35
Rv3252c		Transmembrane alkane 1-monooxygenase alkB	5.41
Rv0972c		acyl-CoA dehydrogenase fadE12	0.26
Rv0973c		Acetyl-/propionyl-CoA carboxylase alpha subunit accA2	0.22
Rv0974c		Acetyl-/propionyl-CoA carboxylase beta subunit accD2	0.24
Rv0975c		acyl-CoA dehydrogenase fadE13	0.26
Rv1344		acyl carrier protein	0.30
Rv2941		Fatty-acid-CoA ligase fadD28	0.31
	PE/PPE family		
Rv1168c		PPE family protein, PPE17	7.23
Rv1169c		PE family protein, PE11	10.80
Rv0754		PE-PGRS family protein, PE_PGRS11	0.30
	Regulatory protein		
Rv0165c		Transcriptional regulator, gntR-family, mce1R	4.8
Rv0232		Transcriptional regulator, tetR/acrR-family	3.73
Rv0792c		Transcriptional regulator, gntR-family	4.29
Rv2621c		Transcriptional regulator	6.36
Rv2912c		Transcriptional regulator, tetR-family	4.26
Rv2989		Transcriptional regulator	3.03
Rv3060c		Transcriptional regulator, gntR-family	3.63
	Virulence, detoxification and adaptation		
Rv1397c		Possible toxin vapC10	3.12
Rv1398c		Possible antitoxin vapB10	3.42
Rv3357		Conserved hypothetical protein	0.30
Rv3358		Conserved hypothetical protein	0.25
Rv2428		Alkyl hydroperoxidereductase C protein ahpC	0.17

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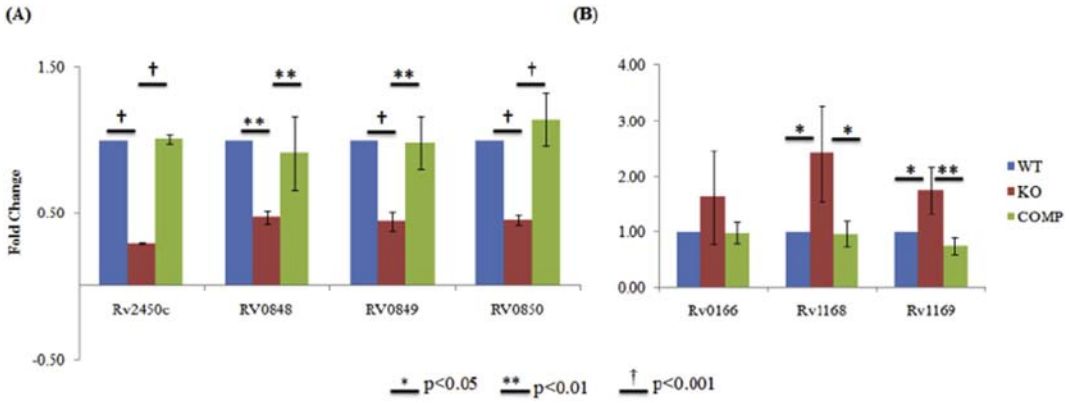

Figure 2. Real time PCR results for a set of genes differentially affected in *groEL1*-KO vs WT under low aeration condition. RT-PCR was performed on RNA isolated from WT, *groEL1*-KO and *groEL1*-Comp cultures subjected to low aeration stress. (A) represents genes down regulated in *groEL1*-KO and (B) represents up regulated genes in *groEL1*-KO vs. WT under low aeration stress. Data are represented as mean change in fold change ± standard deviation for each gene. The statistical significance of data was calculated using One Way Anova in SigmaPlot.

when compared to WT LA (Figure 3). These are: Two Component Systems PhoP/R and DosR/S; Sigma Factors: SigM; and Rv0081, Rv0260c, Rv0328, Rv1332 and Rv3208. Comparison of KO LA with WT LA revealed the SigC regulon to be uniquely affected in *groEL1-KO*; with no changes being observed on comparing WT low aeration to WT log phase gene expression. Significant inverse correlations observed for the regulons common between the *groEL1-KO* and WT (Supplementary Figures 6A and B) are detailed below.

3.1.1.1.1. Two component systems. The two component systems PhoP/R and DosR/S play an important role in maintaining persistent infection in animal models and may have interesting correlation to adaptation to low aeration stress [58–61]. Besides the role in survival in the host, the PhoP/R two component system is also associated with the synthesis of virulence lipids [62], adaptation to acidic pH [63] and positive regulation of DosR expression [39]. Similarly, DosR plays an important role in regulating the switch from aerobic to anaerobic respiration by inducing expression of genes involved in anaerobic respiration (*Rv0081-88*) [64]. Although, regulons of PhoP and DosR were affected in both WT and *groEL1-KO* under low aeration condition, the PhoP target genes *Rv0847 – Rv0850* (also co-regulated by RicR) [65] showed contradictory gene expression changes in the *groEL1-KO* and WT in adaptation to low aeration (Supplementary Figure 7A). Unlike WT that witnessed a 5–12-fold increase in expression of this operon on being subjected to low aeration stress, the expression of *Rv0847-Rv0850* was decreased in *groEL1-KO* (~0.10 log fold change) as compared to WT LA. A similar inverse correlation in genes expression was also observed for PhoP target genes – *Rv3145* and *Rv3148* [65] which had increased expression in KO LA. DosR targets- *Rv3534c-3535c* encoding for acetaldehyde dehydrogenases too had a decreased expression in KO LA as against increased expression in WT LA (Supplementary Figure 7B). The inverse correlation correlated with decreased adaptability of *groEL1-KO* to survive under low aeration stress.

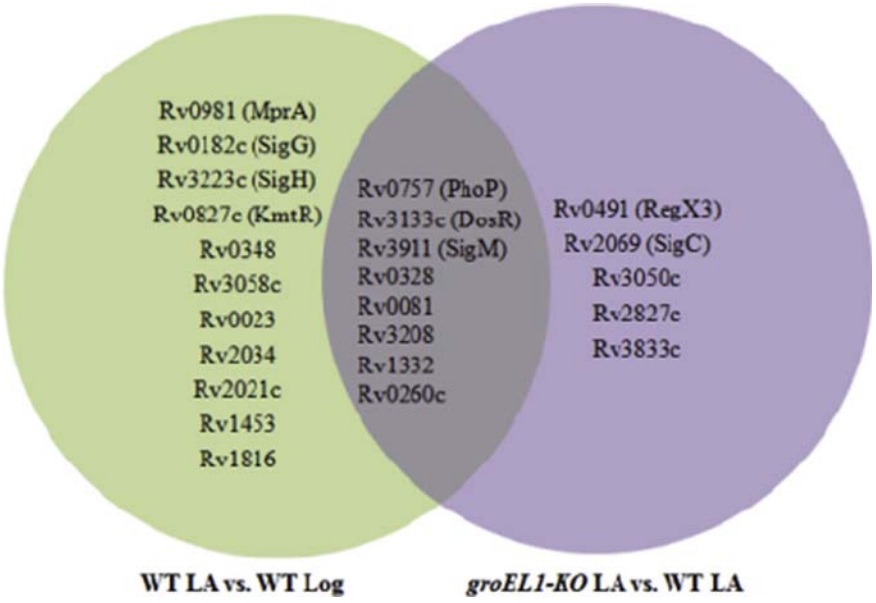

Figure 3. Venn diagram representing the regulons affected on subjecting WT and *groEL1-KO* to low aeration stress. The green circle represents the genes differentially expressed on subjecting WT to low aeration stress in comparison to WT log phase. The purple circle represents the genes differentially affected in *groEL1-KO* vs. WT under low aeration. The intersection represents the genes affected in *groEL1-KO* and WT in low aeration stress. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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3.1.1.1.2. Sigma factors. Of the 13 sigma factors encoded by the Mtb genome, Sig A-M, SigA is the principal sigma factor, while SigC, D, E, G, H, I, J, K, L and M are called as extracytoplasmic function sigma factors. These regulate cell envelope synthesis, protein secretion, folding and degradation [66–68]. The TRN analysis revealed SigM regulon was affected in both WT and *groEL1-KO* under low aeration, while SigC regulon was uniquely affected in the *groEL1-KO* (Figure 3). The importance of SigC in pathogenesis and disease progression can be judged from its orthologs being present in only the pathogenic mycobacterial species [69] and decreased immune pathogenesis of *sigC* deletion mutant in guinea pig and mouse model of infection [70,71]. Comparison of gene expression profiles of SigC target genes for *groEL1-KO* and WT under low aeration condition revealed decreased expression of genes: *Rv2963* (0.11 log fold change), *Rv0967-Rv0969* (0.23–0.30 log fold change), along with decreased *Rv0846* (0.23 log fold change) in the *groEL1-KO* (Supplementary Figure 8A). These genes have been implicated to play a role in maintaining copper homeostasis in the bacterium [41,42,72] which is an important cofactor for enzymes involved in respiration and detoxification like amine oxidases, mono-oxygenases, nitrite reductase and superoxide dismutase [73]. On the other hand, a nearly 3-fold increase was observed for SigM targets: *Rv3612c-Rv3616c* [68] in the KO LA as compared to WT LA (Supplementary Figure 8B). Such an increase in expression of EspA regulon genes: *Rv3612c-3616c* under hypoxia has not been reported yet, and maybe a consequence of absence of GroEL1. Intriguingly, even though the levels of *sigM* and *sigC* gene were unaffected under KO LA, the sub-networks were inversely affected in the TRN studies under WT LA and KO LA respectively (Supplementary Figure 8).

3.1.1.1.3. Other regulons – Rv0081, Rv0260c, Rv0328, Rv1332 and Rv3208. Of the five regulons, Rv0081, Rv0260c, Rv0328, Rv1332 and Rv3208; Rv0081 and Rv0260c have been previously reported to play a role in adaptation to low oxygen levels. Rv0081 is reported to regulate expression of ~400 genes [39], repressing the expression of its downstream genes *Rv0082 – 88* encoding the only formate hydrogenlyase complex in Mtb. *Rv0081* had a nearly 2-fold decrease in expression in *groEL1-KO* under LA stress. However, we did not observe concomitant up regulation of the *Rv0082-Rv0088* genes, presumably as this operon is also under the control of DosR and MprA regulators [64]. Similarly, a decrease in the expression of Rv0260c targets *Rv1460-66* was observed in the KO LA. Components of this operon encode for an ABC transporter (*Rv1463*) and a probable cysteine desulfurase (*Rv1464*) and nitrogen fixation protein (*Rv1465*). An increase in expression of homolog of Rv1463 in *M. smegmatis* has been reported in adaptation to survival in low oxygen conditions [74]. Although the gene expression for the other three transcription factors *Rv0328, Rv1332* and *Rv3208* was unaffected under low aeration stress, an inverse correlation in the expression of their target genes was observed in the WT LA and KO LA data (Supplementary Figure 9). Of the three, only Rv3208 was essential for *in vitro* growth [75], while Rv1332 is essential for growth in primary murine macrophages [76] and in C57BL/6J mouse spleen [77]. However, the role for Rv0328 is yet to be defined.

4. Discussion

One of the most rampant human pathogens, *M. tuberculosis* is estimated to have infected nearly 2 billion people globally [10]. Various factors that contribute to Mtb pathogenesis include its ability to (i) adapt to host induced stress conditions, (ii) prevent normal phagosome maturation, (iii) persist for years in an asymptomatic state within the host, and (iv) reactivate from latency to active disease. All these factors have made *M. tuberculosis* a difficult pathogen to contain.

GroELs are the components of essential protein folding machinery of the cell as well as important antigens of Mtb. GroEL in *E. coli* carries out folding of substrate proteins into their native structure in an ATP-dependent manner along with the 10 kDa subunit GroES. In *E. coli,* GroEL and GroES fold ~13% of the proteins [78]. While most bacteria have just one copy of GroEL, many members of Actinobacteria, Cyanobacteria and Chlamydia have two or more copies of the *groEL* gene [79–81]. Mtb has two copies, GroEL1 and GroEL2, both of which show increased expression levels during various stress conditions like oxidative stress [14], heat shock [13] and infection [15]. The essentiality of GroEL2 was reported by Hu et al. [19]. Similarly, Ojha et al. [20] showed that GroEL2 is essential in *M. smegmatis.* Additionally, Fan et al. [23] stated that *M. smegmatis* and Mtb GroEL2, and not GroEL1, could replace the essential *groEL* gene in *E. coli* and can function with *E. coli* GroES as well as with Mtb GroES. Thus, the biological function of GroEL1, which appears to be non-essential for growth, appears enigmatic.

Our *in vitro* growth of the *groEL1-KO* confirmed that GroEL1 is dispensable for survival under normal conditions (Supplementary Figure 2). Similarly, the loss of GroEL1 did not affect the ability to survive under certain stress conditions – cold shock and stationary phase. However, we demonstrated an increased vulnerability of the *groEL1-KO* to low aeration stress. In low aeration/standing cultures [82], a gradient of oxygen concentration is formed. A 40% decrease in CFU could be observed in *groEL1-KO* relative to WT cultures even at 24 h. Further, when this condition was made more stringent by adding paraffin oil to the standing cultures and measuring growth over a 7 day time period, a significant drop in growth was observed for *groEL1-KO* at day 1 and day 7. Thus, GroEL1 might have an important role to play for adaptation under low aeration conditions.

Availability of oxygen is one of the most important parameters affecting cell growth. Cell modulates its carbon and electron flow pathways depending on the presence of molecular oxygen in the environment. Mtb, an obligate aerobe, in absence of oxygen or limited availability of oxygen switches to alternate electron acceptors like nitrate, nitrite etc. These alternate electron acceptors help in maintaining the $NADH/NAD^+$ ratio, thus maintaining redox balance of the cell. Under limited oxygen availability, the *groEL1-KO* witnessed decreased expression of formate hydrogen lyase complex (*Rv0081-88*) an operon implicated for its role under dormancy [39] and on subjecting cells to nitrate stress [64]. Additionally, cofactors and co-enzymes that are known to be a vital part of enzymatic reactions under anaerobic conditions were too decreased in the *groEL1-KO* under low aeration stress. Transcriptomic analysis of *groEL1-KO* under low aeration revealed down regulation of genes in copper response and genes encoding members of folate synthesis super pathway. Copper is an important metal cofactor in redox enzymes such as oxidases and superoxide dismutases [73] and as the terminal electron acceptor in cytochrome *c* oxidase [83] making it an essential element for maintaining cell viability. Similarly, folate is an important cofactor in the synthesis of protein and nucleic acid backbone of the cell (purines, pyrimidines; amino acids), besides playing a role in maintaining redox homeostasis. TRN analysis revealed a suppression of response in *groEL1-KO* for key regulons (Figure 4) required for survival under low aeration condition. We propose that decreased ability of *groEL1-KO* to survive under low aeration stress may be due to the reduced ability of *groEL1-KO* to oxidize reduced equivalents of NAD^+ and inhibition of enzymes due to limited availability of cofactors. Thus, although GroEL1 is not essential under normal

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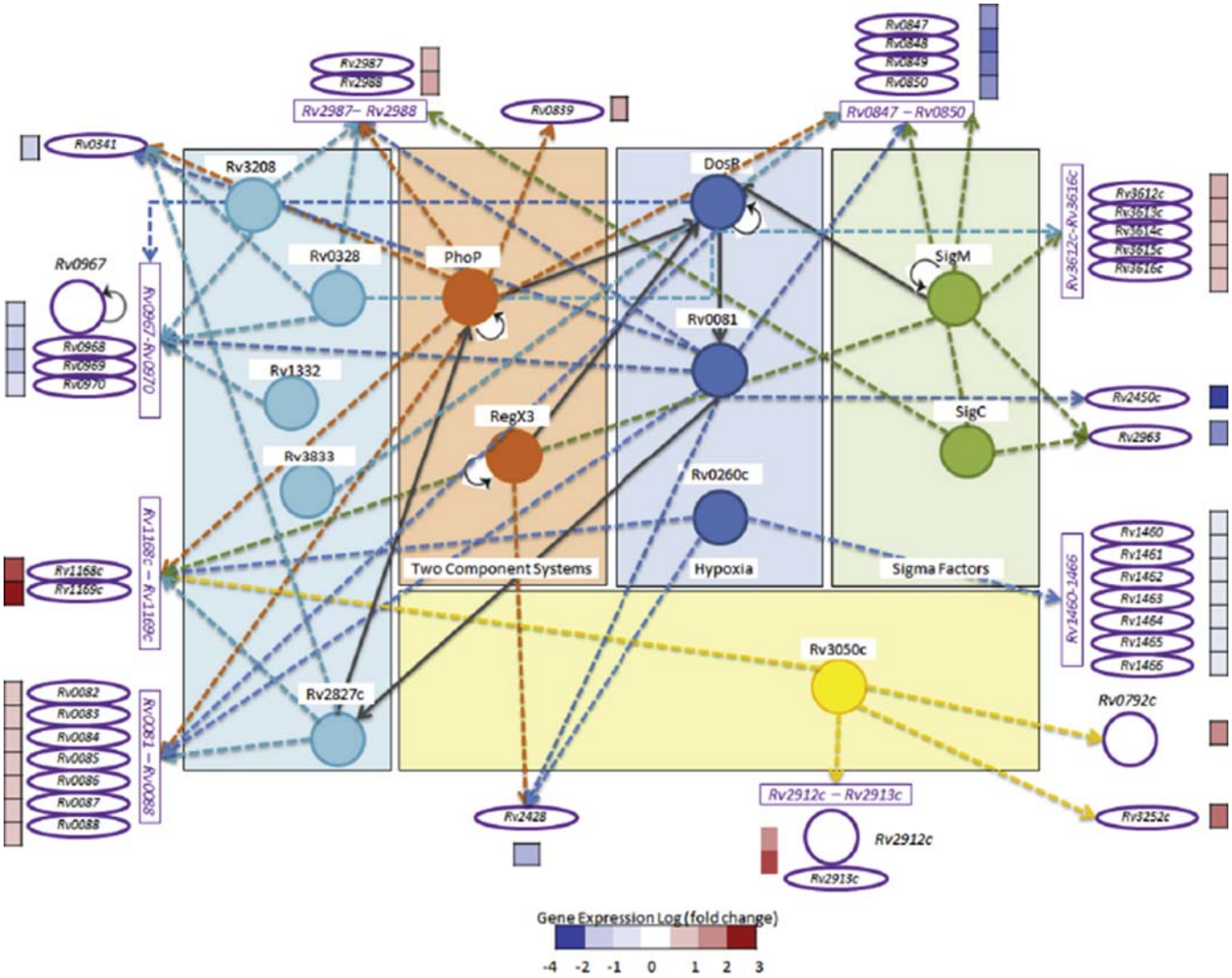

Figure 4. Transcription factor (TF) regulatory network representing selected regulatory interactions that highlight the responses to low aeration stress in *groEL1-KO*. Circles represent TFs and ovals represent target genes. Interactions between TFs are represented with solid grey lines, between TFs and target genes by dashed lines, and self-regulation of TFs with black lines. Different transcription factors are represented by different colours depending on the category they belong to: green – sigma factors, dark blue – TFs having a role in hypoxic response, orange – TFs belonging to two component systems, yellow – TFs with a role in macrophage infection, and light blue – undefined category. The coloured boxes next to the target genes represent log fold change in gene expression for the *groEL1-KO* vs. WT under low aeration stress. Note: The dashed lines showing interactions between TFs and their target genes are coloured as per the category of the TF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conditions, it may have a more active role in adaptation and survival under low aeration stress.

Mtb is known to survive in host cells for long intervals of time. We observed that the absence of GroEL1 did not affect the ability of *groEL1-KO* to survive in THP1 cell line. Similar results were also reported by Hu et al. [19] for survival of the *groEL1-KO* in bone marrow derived macrophages or in J774A.1 cell line. However, they observed that the *groEL1-KO* had diminished immunopathology in host lungs and spleen, although the bacterial load of the *groEL1-KO* was the same as the parent strain. Apart from the role of GroEL1 as a potent cytokine inducer [26,27], the attenuation of immune response could also be due to decreased expression of key virulence factors. A similar decreased immunopathology has also been reported for copper responsive proteins CtpV [44], sigma factor SigC [71,84] and two component system DosR/S [85]. Hereby, we propose that the impaired pathology may be a consequence of decreased expression of genes important for virulence.

With this study we could conclude that GroEL1 plays significant physiological role in helping Mtb survive low aeration, a condition faced by Mtb in the hostile acellular environment of granuloma. It is currently unclear if the changes observed in the gene expression in *groEL1-KO* are a consequence of the DNA binding activity or protein folding ability of GroEL1; we are looking at both the possibilities. GroEL1 is not an essential chaperone in nutrient rich (7H9 media) log phase conditions, however, it might interact with a subset of substrates and/or interacting partners under stress conditions. Further, ChIP-Seq experiments under log phase and low aeration will likely reveal its role as the DNA binding chaperone.

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Competing interests: None declared.

Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2015.11.003.

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