Deletion of the *rel* gene in *Mycobacterium smegmatis* reduces its stationary phase survival without altering the cell-surface associated properties

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Dormant or latent physiology of the mycobacterial species is a subject of current investigation in order to understand the long-term persistence of these organisms inside the host. It is argued that the carbon-starved mycobacteria may serve as a good model for the dormant bacilli. The *relA/spoT* gene is upregulated during carbon starvation in *Mycobacterium tuberculosis* and the deletion of the gene resulted in reduction of long-term persistence in *M. tuberculosis*. Over-expression of the gene in *M. smegmatis* changes the colony morphology of the bacteria. Here we show that knock-out of the *relA/spoT* gene compromises stationary-phase survival of *M. smegmatis*. However, the Δ*relA/spoT* bacteria show similar profile of glycopeptidolipids as the wild-type bacteria under carbon starvation. We have seen here that *M. smegmatis*, a non-pathogenic species, upon carbon starvation exhibits reduced association with murine macrophage cell line RAW 264.7 in comparison to *M. smegmatis* grown in carbon-enriched medium. But the clearance of the bacteria from macrophages takes place in the same window of time in both conditions.

MYCOBACTERIUM SMEGMATIS is a fast-growing, non-pathogenic species of mycobacteria. Due to its short doubling time, *M. smegmatis* has always been argued to be a suitable model for studying the slower-growing pathogenic mycobacteria. But as the species lacks the determinants of mycobacterial virulence, the design of experiments and interpretation of results require careful control. On the other hand, the stationary-phase physiology and the survival of the organism for extended periods of time are interesting phenomena, which need to be addressed separately. Long-term persistence of *M. tuberculosis* was first demonstrated by Corper and Cohn[1], following which the anaerobic culture of the bacterium called the Wayne's model of dormancy, gained popularity[2,3]. However, Nyka[4] first proposed that an *in vitro* starved culture of mycobacteria exhibited similar morphology and hydrophobicity as *in vivo* persisting bacilli. We decided to look at the long-time survival of *M. smegmatis* in the stationary phase under starved condition in order to understand the stress physiology of this organism and with an aim to correlate it with the persisting pathogens. It was noticed that the passage of the culture into the stationary phase or carbon limitation in the medium induced several genes like *sigF*[5] and *relA/spoT*[6]. These genes have important roles in the long-term survival of *M. tuberculosis*. We reported earlier that carbon starvation in *M. smegmatis* alters the cellular morphology[7], concomitant with the synthesis of a new polar glycopeptidolipid (GPL)[8]. All mycobacteria are internalized by macrophages[9,10], but only the pathogenic ones survive and replicate intracellularly[11]. In this article we address two questions:

(i) Whether deletion of *relA/spoT* gene, which is responsible for the maintenance of the level of ppGpp in the cell, in *M. smegmatis* has any relevance to its survival in the stationary phase.
(ii) Whether the mutant *M. smegmatis* also shows similar cell surface-associated properties like the GPL profile and association with macrophages.

Materials and methods

Bacterial strains and growth conditions

M. smegmatis mc^{2}155 was grown in MB7H9 broth (Difco) or agar supplemented with 0.05% Tween-80 (Sigma) and 2 or 0.02% (w/v) glucose for normal and carbon-limiting cultures respectively, at 37°C. Media for growing the *relA/spoT* mutant of *M. smegmatis* had 25 μg/ml hygromycin (Sigma). Amikacin (Torrent Pharma) was used in the cell association and intracellular survival assay, in order to kill the extracellular bacteria following infection of the cells.

Eukaryotic cell line

Murine macrophage cells RAW 264.7 (ATCC number TIB-71) were grown in Dulbecco's modified Eagle's medium high glucose (Sigma) supplemented with 10% foetal bovine serum (Sigma), hereafter referred to as DMEM-10%, under 5% CO_2 at 37°C. RAW 264.7 cells were obtained from Maneesha Inamdar, JNCASR, Bangalore.

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Cell association and intracellular survival assay

The method was devised similar to the procedure of Ramakrishnan and Falkow[9], developed for *M. marinum* and the murine macrophage cell line J-774.

The RAW 264.7 cells used in the experiments had undergone 19 to 24 passages. Adherent cultures of RAW 264.7 were grown in 25 cm^2 tissue-culture flasks (Greiner) for three days. The cells were dislodged and the released cells were recovered by centrifugation, and resuspended in DMEM-10%. The macrophages were then plated in 48-well tissue-culture plates (Costar) at a density of 1×10^5 cells per well (in 200 µl of medium per well). Cells were grown for a period of 24 h at 37°C under 5% CO_2 prior to the infection.

For preparing the bacterial inoculum, mc^2155 was grown to the mid-log phase in normal growth medium and to the early stationary phase in carbon-limiting medium. The *relA/spoT* mutant was grown in the carbon-limiting medium to the early stationary phase. Bacteria were harvested by centrifugation at 1300 *g* for 10 min. The pellets were washed and resuspended in DMEM-10% at a concentration of 3×10^7 cells per ml. Then 200 µl of this bacterial suspension (with an approximate multiplicity of infection [MOI] of 30) was added to the overnight-grown monolayers of RAW 264.7. Phagocytosis of the bacteria was allowed to proceed for 2 h at 37°C under 5% CO_2. Then the macrophage monolayers were washed twice with DMEM-10% and treated with DMEM-10% containing 200 µg/ml Amikacin for 1 h at 37°C to kill the residual extracellular bacteria. The monolayers were washed again with DMEM-10% and the washed medium was plated for assessing the presence of any residual extracellular bacteria. The wells for assaying the intracellular survival of bacteria were then incubated in a medium containing 20 µg/ml of Amikacin. At various time points after phagocytosis, cells in triplicates of wells were lysed. For lysing the cells, 200 µl of sterile water was added to the wells after removal of the medium. Ten minutes later, the contents of the wells were vigorously pipetted and the lysate was collected. The lysates were plated on 7H9 agar at appropriate dilutions and *M. smegmatis* colonies appearing after two to three days were counted for the intracellular bacterial counts.

The wells which were assayed immediately after treatment with the medium containing 200 µg/ml Amikacin, served as the standard for measuring the number of phagocytosed bacteria. The time at which these wells were assayed was considered time zero. Lysis was carried out at 12, 24 and 48 h post infection.

Targetted replacement of relA/spoT in M. smegmatis with a disrupted copy

A 400 base pair (bp) internal region of *relA/spoT* from *M. smegmatis* was amplified in polymerase chain reaction using degenerate primers designed from a highly conserved region of *relA/spoT* as observed in other bacteria, including *M. tuberculosis*.

The sequences of the primers are:

F1 = 5′GTGTGCACCGCNTANGCNAAGT3′
R1 = 5′GTGTACCAGTCNNTGCACACCAC3′

A genomic library of *M. smegmatis* constructed in λ-ZAP II (Stratagene) vector at *Eco*RI site (a kind gift by William Bishai, Johns Hopkins University, Baltimore) was screened by the ^{32}P labelled 400 bp probe. Three plaques which showed positive signals were lifted from the plate and the recombinant phages from the three plaques were excised into recombinant pBluescript SK phagemid with the DNA of interest according to the earlier published method[12]. All the three recombinant plasmids were found to contain 4.2 kb *Eco*RI fragment and were then subjected to automated DNA sequencing to confirm that the three clones had the same origin. One of three clones, pRelMs, was used for further study. pCK0686 (a kind gift from Prof. William Bishai) was used as a suicide vector for disrupting the *relA/spoT*. The vector has *OriE* and kan^r, suc^s, amp^r as a marker on the plasmid. Multiple cloning sites flanked another marker hyg^r on its either side and hence this marker was used for making the disruption construct.

The plasmid pRelMs was digested with *Mlu*I, end-filled with DNA polymerase I (klenow fragment), redigested with *Sma*I and self-ligated to generate a pBluescript SK with 584 bp from the start codon of *relA/spoT*. This N-terminal region was released with *Eco*RI–*Not*I and ligated to pET21b at *Eco*RI–*Not*I site. This extra step of subcloning was carried out to gain an *Nde*I site from pET21b (located upstream of *Eco*RI). Then the subcloned fragment was released with *Nde*I–*Not*I and ligated to *Nde*I–*Not*I of pCK0686. The resulting plasmid pCKNT was used for cloning the DNA fragment downstream to the gene. Similarly, the 1.8 kb fragment downstream to the stop codon of *relA/spoT* was cloned on the other side of the hyg^r. In order to do this, pRelMs was digested with *Mlu*I, end-filled with DNA polymerase I (Klenow fragment), redigested with *Eco*RV and self-ligated to generate pBlueScript SK with 1.8 kb DNA fragment downstream to the stop codon of *relA/spoT*. The 1.8 kb C-terminal fragment was released with *Eco*RI–*Kpn*I, subcloned into *Eco*RI–*Kpn*I site of pMV261. This extra subcloning step was carried out to gain an appropriate cloning site. Then the downstream fragment was released by *Xho*I and subcloned into the *Xho*I site of pCKNT. Thus the resultant plasmid pΔrelMs had a disrupted *relA/spoT*, which was replaced by hyg^r between the 584th and 2423rd nucleotide sequence.

The disrupted copy of *M. smegmatis relA/spoT* placed on pΔrelMs was electroporated into *M. smegmatis*, mc^2155, following the protocol described by Jacobs *et al.*[13], using BIO-RAD electropulsator at 1.5 kV/mm. After 3 h of incubation in antibiotic free medium, the

recombinant clones (mainly due to single crossover) were selected on MB7H9 agar with 2% glucose and 25 µg/ml hygromycin. All the hygromycin-resistant clones were grown in a liquid broth (MB7H9 + 2% glucose + 0.05% Tween-80) till saturation and then plated on MB7H9 agar + 2% glucose + 2% sucrose and hygromycin. The colonies obtained were screened for kanamycin sensitivity, as mentioned earlier. The hyg^r, suc^r and kan^s colonies thus obtained were subsequently confirmed for Δ*relA/spoT* either by Southern hybridization (genotype) or by their failure to accumulate ppGpp (phenotype).

The hybridization protocol was followed according to Sambrook *et al.*[14], except that the hybridization temperature was maintained at 65°C. The protocol described by Ojha *et al.*[7] was used to assay the failure of the mutant to accumulate ppGpp under carbon stress.

Analysis of the cell surface GPL of M. smegmatis

GPL profile of the cell surface of *M. sme*gmatis was carried out as reported earlier[8].

Results

Construction of a functional knock-out of relA/spoT in M. smegmatis

A large portion of *relA/spoT* gene (from 521st nucleotide to the stop codon) in 4.2 kb DNA fragment was replaced by hyg^r cassette. The genotype of the mutant was confirmed by the loss of a *Bgl*II site in the disrupted gene. A Southern hybridization of *Bgl*II-digested genomic DNA of the mutant clones clearly shows the loss of 1.4 kb fragment when probed with an intact *relA/spoT* gene (Figure 1 *a*). This confirmed that the homologous recombination between the chromosome and the disrupted *relA/spoT* occurred at the expected locus and thus mutant clones were Δ*relA/spoT*.

The most important step in phenotypic characterization of the mutant will be a confirmation of failure to accumulate ppGpp under carbon limitation. Figure 1 *b* shows that the mutant fails to produce ppGpp when subjected to carbon starvation. This indicates that the disruption in *relA/spoT* has resulted in the loss of function of the gene.

Stringent pathway is required for long-term survival of M. smegmatis in nutrition-depleted medium

The stringent pathway, when activated by nutritional depletion, helps the bacterium to minimize energy consumption and survive the stressful condition. Therefore, the pathway could be having a role in the long-term survival of *M. smegmatis*, and to test this hypothesis the *relA/spoT* mutant and the wild-type strain of *M. smegmatis* were grown in carbon-limiting (0.02% glucose) MB7H9 and 0.05% Tween-80 till the stationary phase. Then the CFU of both the cultures was determined at

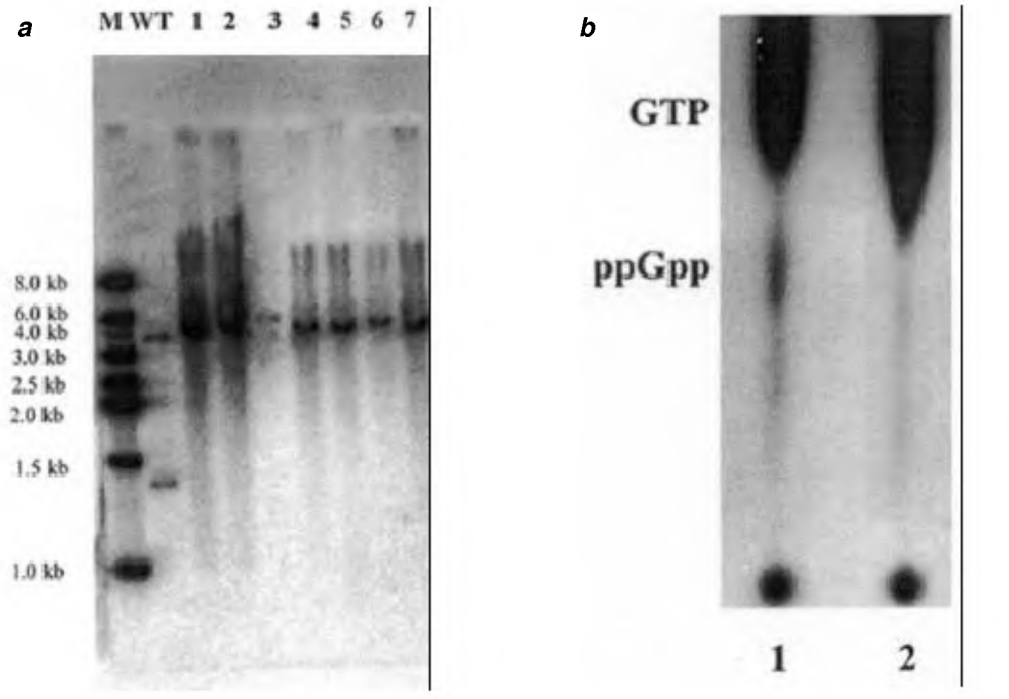

Figure 1. ***a***, Confirmation of genotype of *relA/spoT* mutant using Southern hybridization. The loss of a 1.2 kb *Bgl*II fragment due to double crossover with the disrupted copy of the gene was observed in the mutants. Genomic DNA of each clone was digested with *Bgl*II and probed with 2.4 kb *relA/spoT* DNA fragment. M, DNA ladder; WT, Wild-type strain. ***b***, Confirmation of phenotype of *relA/spoT* mutant. TLC profile of formic acid extract of ^{32}P-labelled carbon-starved culture of the mutant clearly shows the lack ppGpp (lane 2). Lane 1, Formic acid extract of ^{32}P-labelled carbon-starved culture of wild type.

regular time intervals up to 13 days. It can be seen from Figure 2 *a* that within 15 days of its entry into the stationary phase, the culture of the mutant strain has ten fold lesser number of viable bacteria than the wild type. This reduction in viability is not due to the polar effect of the disruption of the *relA/spoT* as when transformed with pMatt2, a single copy plasmid expressing *E. coli relA*, the viability of the mutant strain is restored to the wild-type levels. It should be mentioned at this point that similar results were obtained earlier from a Δ*relA/spoT* strain of *M. tuberculosis*[6]. We have recently reported that a new species of polar GPL is synthesized during carbon starvation in *M. smegmatis*, concomitant with the appearance of a smooth cell surface[8]. Figure 2 *b* shows that the polar GPL is still detectable under the Δ*relA/spoT* condition.

M. smegmatis grown under carbon-enriched as well as carbon-limiting environment has the same temporal profile of survival inside RAW 264.7 cells

Although *M. smegmatis* has been shown to survive within macrophages for a short while, as can be expected for a non-pathogenic strain of mycobacterium, it eventually gets cleared from the macrophages[9,15]. However, as the surface GPL profile of the bacterium changes upon starvation[8], the survival pattern of the bacterium within the phagocytic cells, macrophages, was investigated. But the wild-type *M. smegmatis* was found to be getting cleared from the macrophages within the same window of time, whether grown in a carbon-enriched medium or a carbon-limiting one (Figure 3 *a*). It did not escape our notice that the apparent rate at which the bacteria were cleared was different in the two cases, as the number of bacteria associating with the cells was consistently more in the case of the normal culture. Since complete clearance was achieved by the cells within the same period of time in both the cases, even in experiments where the MOIs varied considerably, this point was not probed further.

M. smegmatis when grown under carbon-limiting medium associates with macrophages at a lesser level than when grown in normal medium

Another aspect we looked into was the degree of initial association of bacteria with macrophages following infection. The CFUs of the time-zero cell lysates denote the number of bacteria initially associating with the macrophages. The number of viable bacteria in the inoculum was calculated by counting the CFUs of serial dilutions of the bacterial inoculum. The percentage of inoculum of bacteria associating with the cells was calculated from these values. The percentage of association was compared between bacteria cultured in the presence of 2% glucose and those grown in carbon-limiting (0.02% glucose) environment. The level of association of the latter was found to be less than one-fifth of the former, when the assay was carried out with nearly identical MOI (Figure 3 *b*). When similar experiments were carried out with the Δ*relA/spoT* mutant under carbon-limiting conditions, no change in this pattern of reduced association with macrophages was found (data not shown).

Discussion

We have looked into the growth characteristics of *M. smegmatis* under the condition when the *relA/spoT* gene is deleted. We observed that the survival of the mycobac-

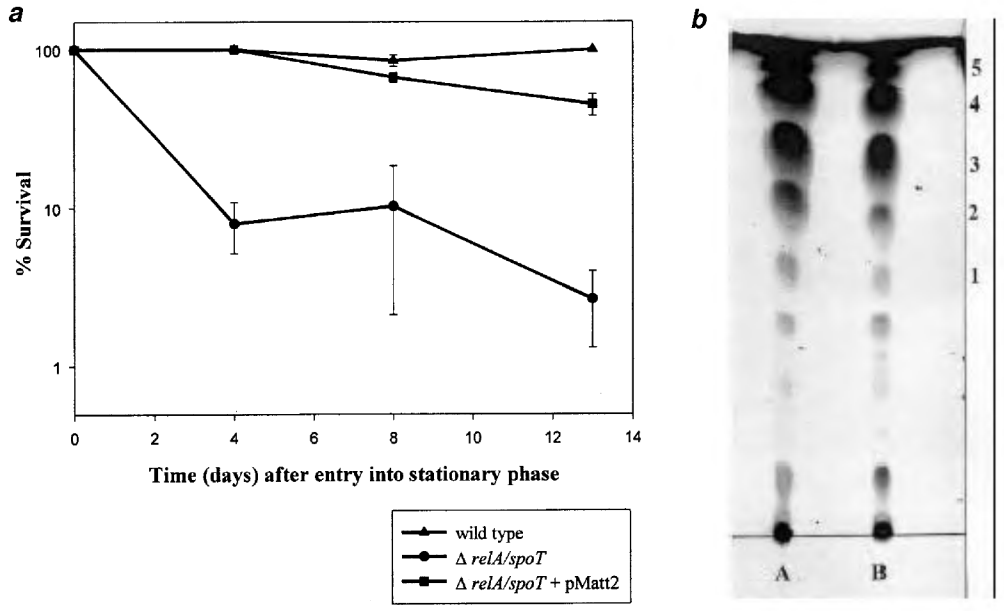

Figure 2. ***a***, Stringent pathway plays an important role in the long-term survival of *M. smegmatis*. CFUs of the cultures were obtained at different time intervals after their entry into stationary phase. ***b***, Synthesis of polar GPL in carbon-starved *M. smegmatis* is not dependent on the stringent pathway. TLC profile of GPL from carbon-starved culture of wild type (A) and *relA/spot* mutant (B) shows the presence of polar GPL (spot 1).

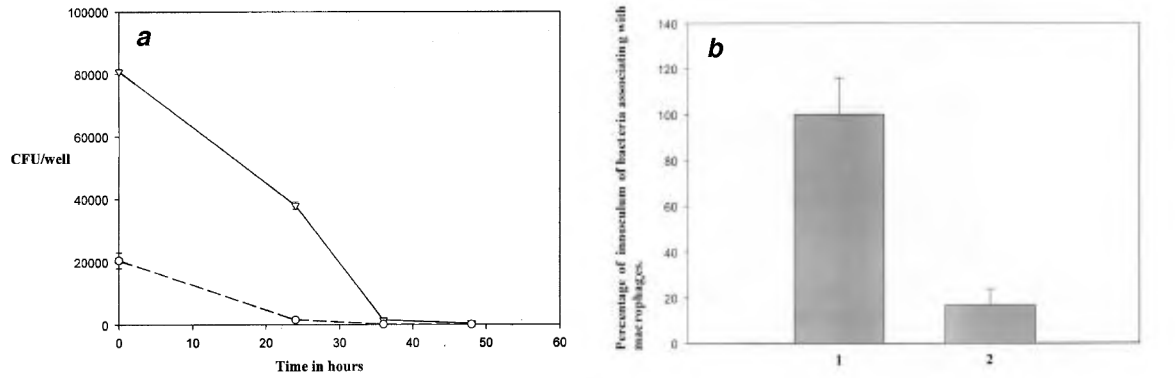

Figure 3. ***a***, Clearance of *M. smegmatis* grown in normal (2% glucose; solid line) and carbon-limiting (0.02% glucose; dashed line) cultures from RAW 264.7 cells showing intracellular bacterial counts coming down to baseline within the same period of time. Representative of three independent experiments. Each point is the mean of two values with the standard error. ***b***, Effect of carbon starvation on initial association of *M. smegmatis* with macrophages. Percentage of inoculum associating with cells in the case of culture grown in 2% glucose is shown as 100% for reference (standard deviation 15.78). The actual value corresponding to 100% was 15,200 CFUs. The value for 0.02% culture is shown as a proportion of association in the case of culture grown in 2% glucose – 16.59% (standard deviation 7.1). Bar 1, mc^2155 grown in the presence of 2% glucose; bar 2, mc^2155 grown in the presence of 0.02% glucose. Each value represents the mean of three values with standard deviation.

teria for a long time is remarkably compromised under such a situation. However, *relA*/*spoT*, the gene controlling the stringent response appears to have no effect on the synthesis of polar GPL, which is specifically induced in *M. smegmatis* under carbon limitation. Interestingly, carbon-limited *M. smegmatis* showed reduced association with macrophages, which perhaps could be related to the altered cell-surface characteristics.

It has been reported that a number of bacilli remain dormant extracellularly in the caseous material within the granuloma[16]. It appears that the change in cell surface of *M. smegmatis* upon starvation[8,17] may play a direct role in its reduced association with macrophages. We did attempt to carry out the experiments with a mutant of *M. smegmatis* in which a peptide synthetase gene involved in the biosynthesis of GPL was mutated[18]. The extreme susceptibility of the mutant to clump in liquid cultures was a hurdle in preparation of a proper bacterial inoculum as well as quantification of the number of bacteria.

The alterations in cell surface as a result of change in the growth environment can be seen as a part of the adaptations that the bacteria undergo to a general stress. Such a general survival strategy can be envisaged to be shared by both pathogenic as well as non-pathogenic bacteria.

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