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High Intracellular Level of Guanosine Tetraphosphate in *Mycobacterium smegmatis* Changes the Morphology of the Bacterium

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Almost one-third of the world population today harbors the tubercle bacillus asymptomatically. It is postulated that the morphology and staining pattern of the long-term persistors are different from those of actively growing culture. Interestingly, it has been found that the morphology and staining pattern of the starved in vitro population of mycobacteria is similar to the persistors obtained from the lung lesions. In order to delineate the biochemical characteristics of starved mycobacteria, *Mycobacteria smegmatis* was grown in 0.2% glucose as a sole carbon source along with an enriched culture in 2% glucose. Accumulation of the stringent factor guanosine tetraphosphate (ppGpp) with a concomitant change in morphology was observed for *M. smegmatis* under carbon-deprived conditions. In addition, *M. smegmatis* assumed a coccoid morphology when ppGpp was ectopically produced by overexpressing *Escherichia coli relA*, even in an enriched medium. The *Mycobacterium tuberculosis relA* and *spoT* homologue, when induced in *M. smegmatis*, also resulted in the overproduction of ppGpp with a change in the bacterium's growth characteristics.

Mycobacteria have emerged as a major threat to humankind, for as many as one-third of the world's population (1.7 billion) harbors the tubercle bacillus asymptomatically (18). The latent bacilli can persist in a somewhat ill-defined physiological state in pulmonary and extrapulmonary lesions for years after infection (30). These bacilli are opportunistic and can reactivate themselves when the host is immunocompromised. To add to the misery, persistors require prolonged therapy, and *Mycobacterium bovis* BCG vaccination has little effect in blocking reactivation of the bacteria. Hence, for improved control of tuberculosis, it is imperative to develop effective drugs to cease the propagation and persistence of these latent bacilli. This will be greatly facilitated by a better understanding of the physiological state of these latent bacteria. Although several in vitro models suggest low extracellular concentrations of oxygen to be an important cause for mycobacterial dormancy (8, 10, 42), the effect of this state on cellular metabolism is not clear.

It has been shown that the morphology and staining pattern of an in vitro culture of mycobacteria differ from those of persistors which are obtained from the lung lesion and are chromophobic to the conventional acid-fast staining (25, 26). The former is an acid-fast and long rod-shaped bacillus, as opposed to the latter, which is non-acid fast and granular. However, these persistors can be stained after oxidizing the cell surface with periodic acid. In yet another important observation, it has been shown that the morphology and staining pattern of such persistors can be obtained in vitro by starving the *Mycobacterium tuberculosis*, *Mycobacterium kansasii*, or *Mycobacterium pheli* cultures on agar plates without any nutrients (26). This key observation suggests that the natural persistors may be physiologically similar to bacteria in nutritionally starved cultures. Thus, studying the physiology and morphology of such starved cultures could provide some important clues towards understanding the mechanism of latency. This hypothesis prompted us to take up the study of starving mycobacteria.

Bacteria adapt to nutritional stress for their survival, predominantly through a mechanism termed the stringent response. The hallmark of the stringent response is the accumulation of guanosine tetraphosphate (ppGpp), also called the stringent factor, and downregulation of stable RNA (rRNA

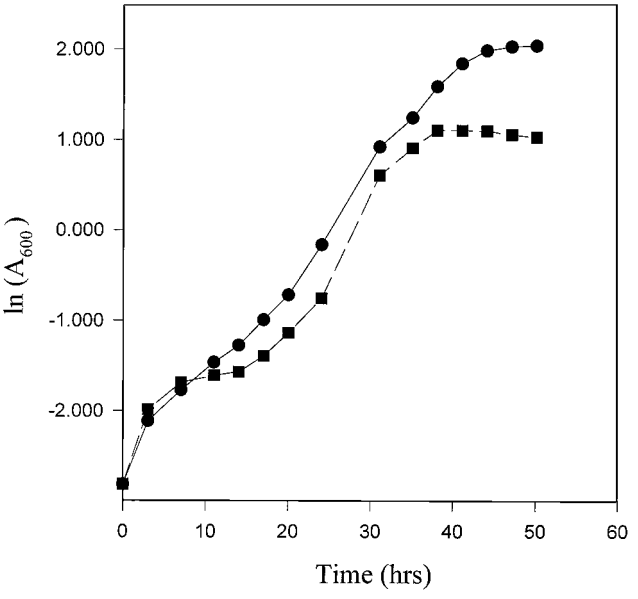

FIG. 1. Comparative growth kinetics of normal (●) and carbon-starved (■) cultures of *M. smegmatis*. The generation time of a normal culture grown in enriched medium (2% glucose) is 2.0 h, whereas that of a carbon-starved culture (0.2% glucose) is 2.8 h. An early entry into the stationary phase at low cell density of the latter is evident from the profile.

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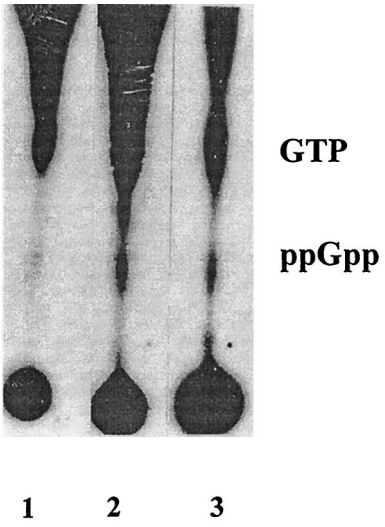

FIG. 2. Accumulation of ppGpp in carbon-starved *M. smegmatis*. Five microliters of ^{32}P-labeled formic acid extract of normal culture grown in MB7H9–2% glucose–0.05% Tween 80 (lane 1) and carbon-starved culture grown in MB7H9–0.2% glucose (lane 2) were loaded on a PEI-coated TLC plate and resolved as mentioned in the text. To confirm the authenticity of the spot, a ^{32}P-labeled formic acid extract of *E. coli* strain (CF3120, overexpressing the ppGpp synthase gene, *relA*, was loaded as a control (lane 3).

and tRNA) synthesis (3). It appears that RNA polymerase is the ultimate target of ppGpp (6), although the exact mode of selective downregulation of the gene expression is not clear.

Many bacteria can assume a well-defined physiological state under starvation conditions, which facilitates their survival (23, 27, 38). The role of ppGpp in the developmental process of these physiological states has been a subject of interest for many researchers over the years. It has been extensively studied in *Myxococcus xanthus*, in which accumulation of ppGpp has been observed to be an important requirement for the formation of the fruiting body (16). In *Streptomyces coelicolor*, ppGpp has been implicated in the synthesis of antibiotics in the stationary phase of the bacteria (5). Though ppGpp has been detected in various other prokaryotes during starvation, e.g., *Bacillus subtilis* (28), *Bacillus stearothermophilus* (12), *Staphylococcus* spp. (4), *Streptococcus equisimilis* (24), and *Salmonella enterica* serovar Typhimurium (20, 35), its function in these organisms is yet to be assigned.

Although *Mycobacterium smegmatis* is nonpathogenic, it shares many biosynthetic pathways with *M. tuberculosis* and may serve as a good model system. In addition, its higher growth rate makes it a suitable candidate for starvation studies. In this study we have shown that ppGpp accumulation is accompanied by morphological change in *M. smegmatis* under carbon starvation conditions. Furthermore, we have shown that *M. smegmatis* assumes the coccoid morphology (similar to the persistors) when ppGpp is ectopically produced by overexpression of *Escherichia coli relA* in an enriched nutritional medium. We have also characterized the in vivo function of the *M. tuberculosis relA*/*spoT* homologue in *M. smegmatis*.

MATERIALS AND METHODS

Strains, media, and growth. *M. smegmatis*, strain mc^{2}155, was grown in MB7H9 (Difco) with 2% glucose and 0.05% Tween 80 for enriched culture. In the carbon-starved medium the glucose concentration was reduced to 0.2% without any Tween 80 in the medium. For comparative studies of pMatt1 and pMatt2, samples of the culture stock from −70°C were subcultured once before being inoculated in the experimental culture. For acetamide-induced expression, bacteria were grown in MB7H9 having 2% succinate with 0.05% Tween 80 and the culture was induced with 2% acetamide. For the plate culture the same composition was used with 1.5% agar. The growth kinetics of the culture was studied by measuring the culture's optical density (OD) at 600 nm. *E. coli* strain CF3120 is *relA*-overexpressing strain which bears *relA* under the control of the Ptac-*lacI*^{q} promoter-operator system on a multicopy plasmid, pALS10. The gene is induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to Luria-Bertani medium.

Plasmids. For overexpression of *E. coli relA*, pMV261 (40) bearing Phsp60 was converted into an integrative vector, pMatt1, by removing its *Not*I fragment containing *oriM* and ligating the backbone with the *Sal*I fragment of pDK20 (9), which consists of the integrating signal of mycobacteriophage L5. Then the *Eco*RI-*Hin*dIII *relA* fragment from pALS10 (a gift from Mike Cashel) was subcloned into the *Eco*RI-*Hin*dIII site of pMatt1 to generate pMatt2, thus generating a transcriptional fusion of *relA* with Phsp60.

The *M. tuberculosis relA*/*spoT* homologue (Rv2583c) along with its ribosome binding site (RBS) was obtained from the *Kpn*I-*Eco*RV fragment of the cosmid pY227 (7) and subcloned into the *Kpn*I-*Xba*I (end-filled with Klenow fragment) ends of pAGAN90 (29). The recombinant plasmid, pMtrel2, has an acetamide-inducible 2.2-kb transcriptional apparatus fused to the RBS and open reading frame (ORF) of the gene.

ppGpp detection. A 25-ml *M. smegmatis* culture was grown to an OD of 0.2, and then [^{32}P]H_3PO_4 (BRIT, Hyderabad, India) was added to it to a final concentration of 100 μCi/ml. The labeled cells were harvested at an OD of 0.8, washed once with 10 mM Tris (pH 8.0), resuspended in 50 μl of buffer, treated with 1 mg of lysozyme per ml on ice for 20 min, and lysed with 1% sodium dodecyl sulfate (SDS), and ppGpp was extracted with an equal volume of 2 M formic acid. After centrifugation at a high speed at a cold temperature for 10

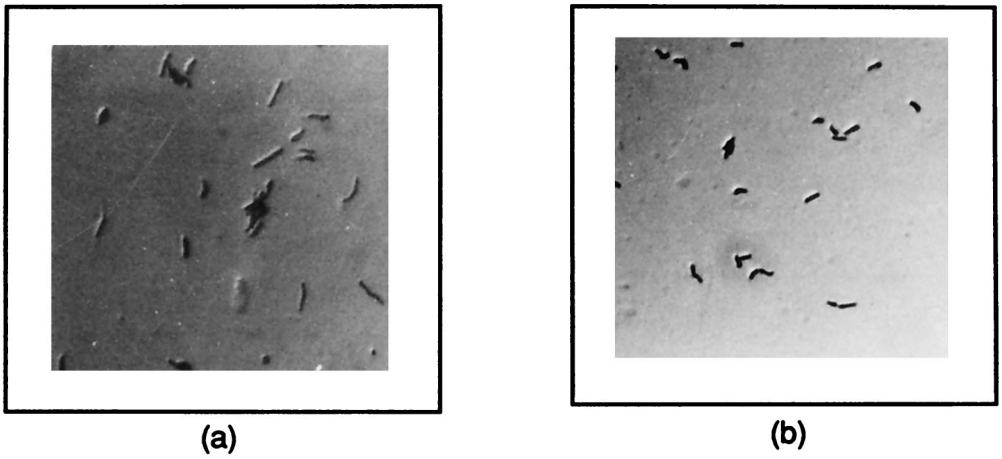

FIG. 3. Morphological difference between normal (a) and carbon-starved (b) cultures of *M. smegmatis*. The two cultures were grown in the same way as mentioned in the legend for Fig. 2. The heat-fixed smear of the cells was stained with carbol-fuchsin and observed under a light microscope in the phase-contrast mode at ×1,000 magnification.

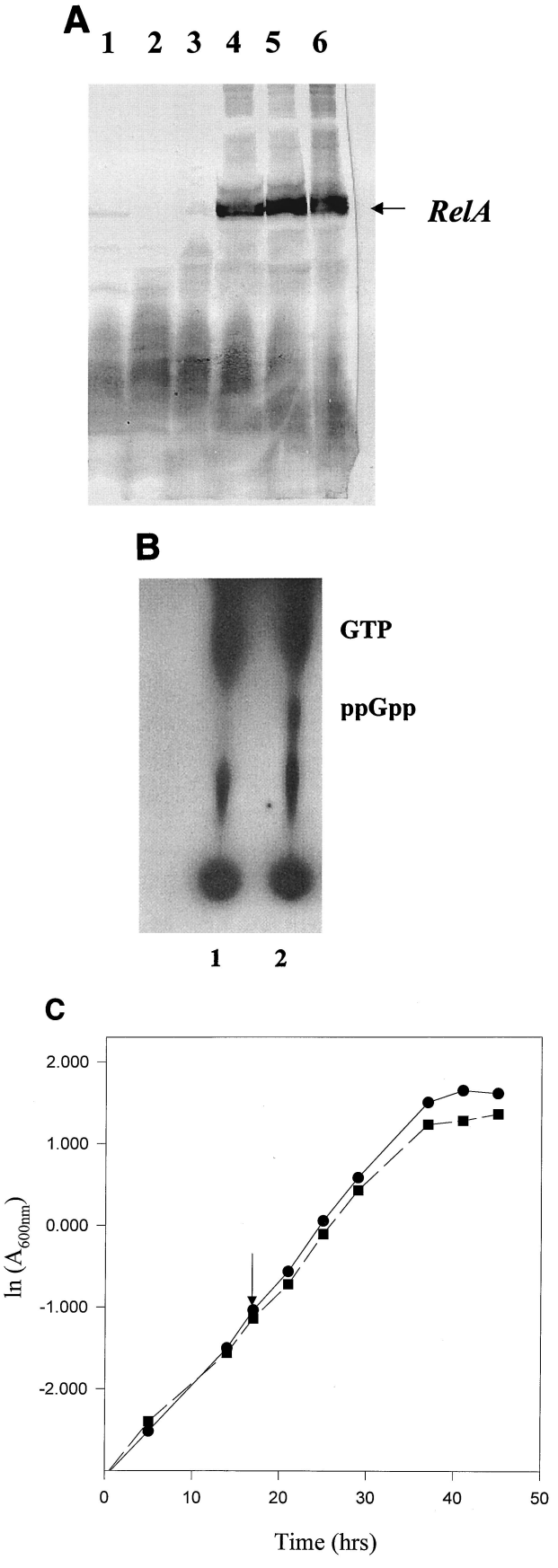

FIG. 4. (A) Expression of *E. coli relA* in *M. smegmatis*. Equal amounts of protein from whole-cell lysate of pMatt1 (lanes 1 to 3) and pMatt2 (lanes 4 to 6) grown at 30°C (lanes 1 and 4), 37°C (lanes 2 and 5), and 44°C (lanes 3 and 6) were subjected to SDS–8% PAGE, transferred to nitrocellulose, and probed with anti-*E. coli relA* antibody. (B) Accumulation of ppGpp upon overexpression of *E. coli relA* in *M. smegmatis*. The strain overexpressing *relA*, pMatt2, was induced along with the control strain, pMatt1, by shifting the culture (at an OD of 0.4) from 30 to 44°C. Five-microliter aliquots of ^{32}P-labeled formic acid extract of pMatt1 (lane 1) and pMatt2 (lane 2) were loaded on PEI-coated TLC plates, and spots were resolved as mentioned in the legend for Fig. 2. Comigration of purified cold ppGpp (not in picture) confirmed the labeled ppGpp spot. (C) Effect of high intracellular levels of ppGpp on growth kinetics of *M. smegmatis*. The generation time of the strain overexpressing *E. coli relA*, pMatt2 (■), was 2.6 h, compared to 2.1 h for the control, pMatt1 (●). Both the cultures were grown in MB7H9–2% glucose–0.05% Tween 80 at 30°C till mid-log phase and were induced by shifting to 44°C. The arrow indicates the time of induction.

min, 5 μl of the supernatant was loaded on a polyethyleneimine (PEI)-coated thin-layer chromatography (TLC) plate (Merck). The plate was developed in 1.5 M KH_2PO_4 (pH 3.4) in one dimension. It was air dried and exposed to X-ray film (Konika) for 18 to 24 h at −70°C for autoradiography. For alkaline hydrolysis of ppGpp, the formic acid extract was immediately neutralized with NH_4OH, treated with 0.3 M KOH, and then treated with 0.1 M $BaCl_2$ at 37°C for 1 h.

In order to detect ppGpp in a *relA* or *relA*/*spoT*-overexpressing system, the ^{32}P-labeled culture was induced at an OD of 0.4 and harvested at an OD of 0.8.

Microscopy. The heat-fixed smear of the culture was stained with carbolfucshin (Loba Chemicals, Mumbai, India), washed with 20% H_2SO_4, counterstained with methylene blue (Loba Chemicals), and observed under a light microscope (Olympus) (magnification, ×1,000) in either phase-contrast or bright-field mode.

Complementation analysis. For functional complementation of *M. tuberculosis relA*/*spoT* in *E. coli*, the *relA* strain of *E. coli* (MC4100) was transformed with pMtrel2 and selected on Luria-Bertani agar containing kanamycin (50 μg/ml). The transformants were streaked on glucose M9 minimal agar plus 100 μg of serine, methionine, and glycine (SMG) per ml or glucose M9 minimal agar. The reversion of the *relA* strain to *relA*$^+$ strain was observed by the loss of sensitivity to SMG.

Miscellaneous. All the cloning experiments and immunodetection were carried out as described earlier (32). The immunoblot was analyzed by a densitometer (Bio-Rad). DNA was electroporated into *M. smegmatis* using a Bio-Rad electroporator at 1.5 kV/mm (17), and transformants were selected on MB7H9 agar containing 20 μg of kanamycin per ml.

RESULTS

Growth kinetics, ppGpp accumulation, and morphology. *M. smegmatis*, strain mc^{2}155, was grown in an enriched (2% glucose) and carbon-deficient (0.2% glucose) medium. The MB7H9 medium contains L-glutamic acid, which can be used as a carbon source for *M. smegmatis*, albeit inefficiently. However, the total carbon coming from 0.2% glucose and glutamic acid was much less in comparison to that from an enriched medium. The bacteria followed altered growth kinetics when grown in carbon-deficient medium. The carbon-deficient culture showed earlier entry into stationary phase at a lower cell density, with an average generation time of 2.8 h, whereas the normal culture, which doubled every 2.0 h, had a very high cell density in the stationary phase (Fig. 1). This observation is consistent with an earlier report on growth kinetics and stationary phase entry of *M. smegmatis* in carbon-limited medium (37). Based in this observation we termed the 0.2% glucose medium as carbon-starved medium for the bacteria.

Following the observation of the growth kinetics the question of whether mycobacteria accumulate ppGpp upon carbon starvation was raised. This question stems from the fact that ppGpp is almost a universal growth regulator in starved prokaryotes. In order to detect the ppGpp accumulation in *M. smegmatis*, ^{32}P-labeled mid-log-phase cells of equal OD (0.8) in enriched and carbon-starved medium were subjected to the extraction procedure (see Materials and Methods). It can be seen from Fig. 2 and comparing lanes 1 and 2 that the formic acid extracts from the cells grown in carbon-deficient medium

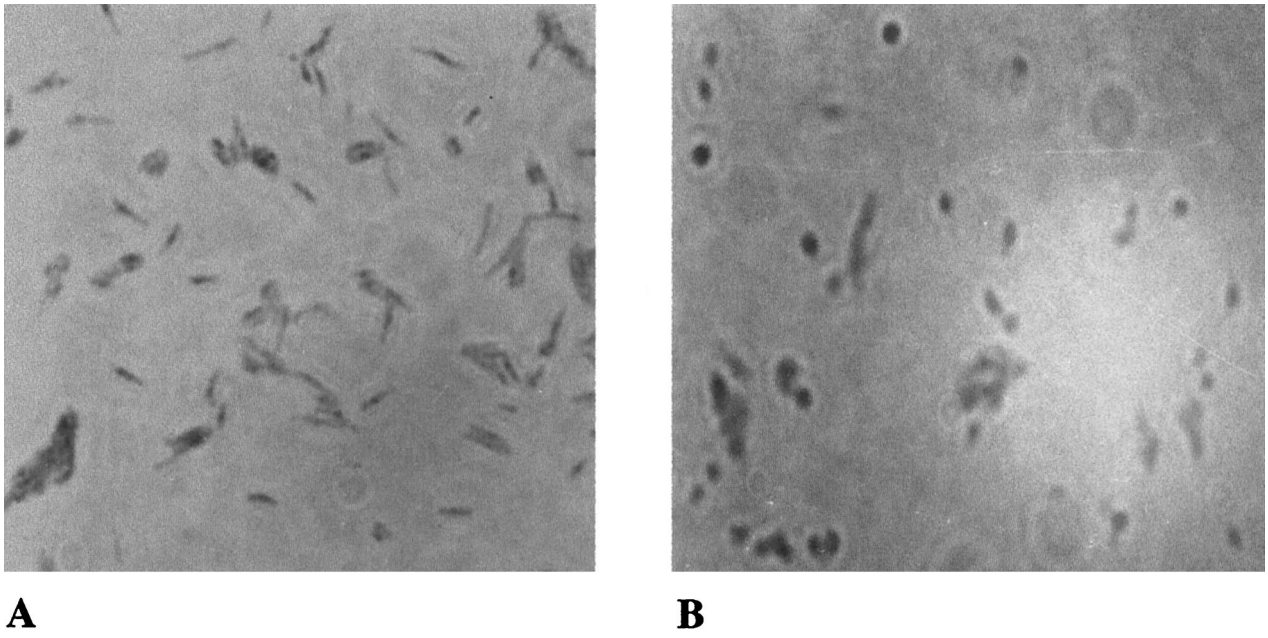

FIG. 5. Morphological difference as a consequence of ppGpp accumulation in *M. smegmatis*. The strain harboring pMatt2 (overexpressing *E. coli relA*) has a spherical morphology (B) compared to the elongated rod shape morphology of the strain carrying pMatt1 (empty vector) (A). The two cultures were grown at 30°C till an OD of 0.4 was reached and then was induced by incubating at 44°C. After 4 h of induction the carbol-fuchsin-stained bacteria were observed with a light microscope in the bright-field mode at ×1,000 magnification.

clearly showed accumulation of ppGpp, in contrast to the cellular extract from enriched medium. The authenticity of ppGpp was demonstrated by its comigration with ppGpp from the ^{32}P-labeled formic acid extract of *E. coli* overexpressing *relA* (CF3120) (Fig. 2, lane 3). The alkaline hydrolysis of ppGpp, as reported earlier (2, 31), was carried out to further confirm the existence of the nucleotide.

In order to compare the morphologies of the normal and carbon-starved bacteria, the acid-fast stains of the two cultures at an OD of 0.8 were prepared and observed under phase-contrast microscope at a magnification of ×1,000. It was observed that the cells under carbon starvation showed reduction in length (almost like a coccoid) in comparison to the normal bacilli (Fig. 3). In order to rule out any effect of Tween 80 on the growth kinetics and morphological changes, the carbon-deficient medium was supplemented with 0.05% Tween 80, and the observations were repeated (not shown). Moreover, the morphology of the bacteria was also observed from the plate culture that was devoid of Tween 80.

Such a morphological change has been reported previously in late-stationary-phase cultures of *M. smegmatis* (37), in which the length of the stationary-phase bacterium is reduced to half. There is indirect evidence to suggest that ppGpp regulates the cell division through FtsZ (a protein required for septum formation) in *E. coli* (41). Overexpression of *relA* in *E. coli* leads to enhanced septum formation and reduced cell size (33). The reduced cell size with concomitant accumulation of ppGpp in *M. smegmatis* suggests a possible role of ppGpp in the morphological changes in mycobacteria.

Overexpression of *E. coli relA*. In order to understand the correlation between morphological changes and accumulation of ppGpp, we tried to overexpress *relA* (ppGpp synthase) from *E. coli* in *M. smegmatis*. An attempt to overexpress *relA* from a multicopy plasmid (pMV261) containing the BCG Phsp60 pro-

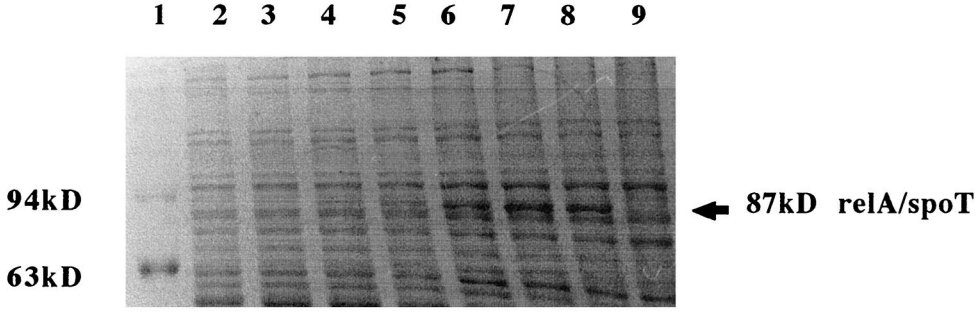

FIG. 6. Coomassie-blue stained polyacrylamide gel showing acetamide-induced expression of *M. tuberculosis relA/spoT* in *M. smegmatis*. The mid-log phase of the culture harboring pMtrel2 was induced with 2% acetamide. A 1-ml aliquot was taken out at the indicated time interval after induction. Equal amounts of the total cellular proteins were resolved by SDS–8% PAGE. Lane 1, marker; lanes 2 to 8, 0, 2, 4, 6, 8, 10, and 20 h, respectively, after induction; lane 9, total cellular protein of a saturated culture of a strain harboring empty vector, pAGAN90.

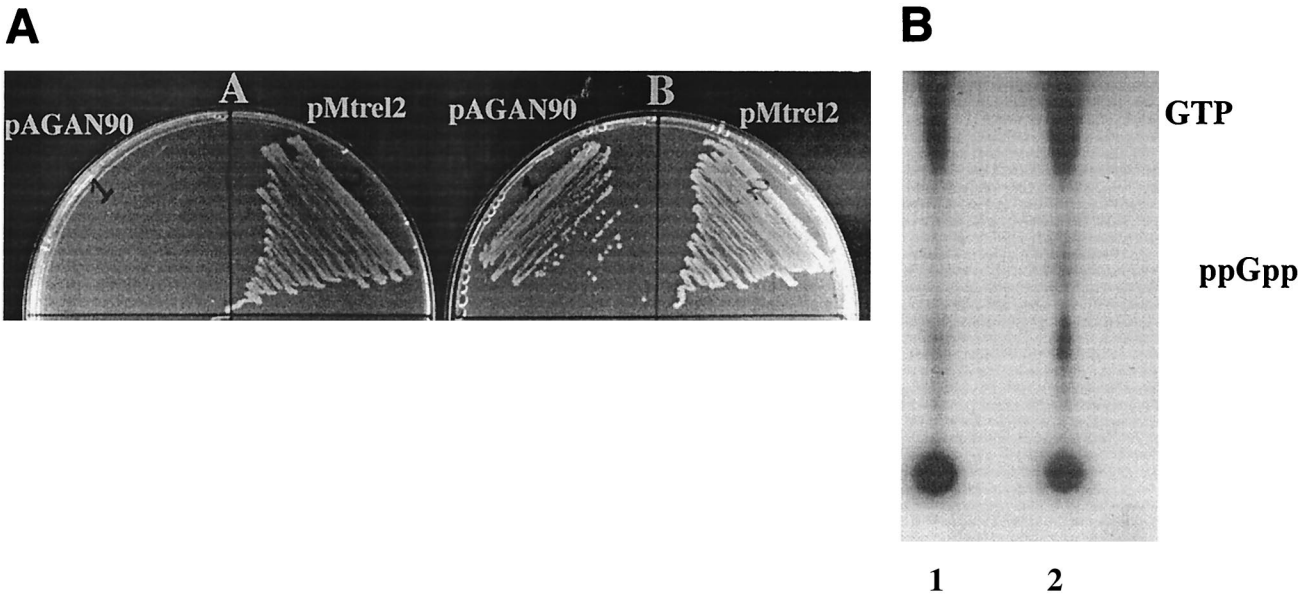

FIG. 7. (A) complementation of *relA E. coli* (MC4100) by the *M. tuberculosis relA/spoT* homologue. The reversion of *relA* to *relA*$^+$ was assayed by loss of SMG sensitivity. The transformant harboring empty vector (pAGAN90) was streaked on the left sector, whereas the one harboring vector with the gene (pMtrel2) was streaked on the right sector. Panel (A) Minimal medium plus SMG; panel (B) minimal medium. (B) ppGpp synthetic activity of the *M. tuberculosis relA/spoT* homologue in *M. smegmatis*. The ^{32}P-labeled cells were grown in MB7H9–2% succinate till mid-log phase and then induced for 4 h with 2% acetamide. Five-microliter aliquots of the formic acid extract of strains pAGAN90 (lane 1) and pMtrel2 (lane 2) were loaded on PEI-coated TLC plates and the spots were developed as described in the text.

moter was not successful. This could be explained by the fact that the gene driven by Phsp60 from a multicopy vector would produce a high level of ppGpp, which would perhaps be toxic for the organism. Since cellular response to ppGpp is dose dependent, a single-copy vector was chosen. An integrative vector (pMatt1) was constructed by replacing *oriM* from pMV261 with *att-int* (the attachment site and integrase protein, from mycobacteriophage L5). A transcriptional fusion of *E. coli relA* and Phsp60 was constructed by subcloning the gene along with its translational signal downstream of Phsp60 in pMatt1 to generate pMatt2. Both pMatt1 and pMatt2 were electroporated into *M. smegmatis*. The expression of *relA* at the translational level was confirmed by immunoblotting against anti-*E. coli relA* antibody (Fig. 4A), and the bands were quantitated using a densitometer. Although the basal level of protein at 30° was high, the expression was temperature dependent. There was an almost 2.5-fold increase in expression upon shifting the culture from 30 to 44°C. However, there was only a 30% increase in protein level when the culture was shifted from 30 to 37°C. Hence, for all the subsequent experiments on pMatt1 and pMatt2 strains the cultures were maintained at 30°C and induced by shifting to 44°C. The intracellular levels of ppGpp in the strains containing pMatt1 and pMatt2 were compared in mid-log phase. As can be seen from Fig. 4B, the strain containing pMatt2 showed accumulation of ppGpp in contrast to the strain containing pMatt1, in which there was no such accumulation. It confirmed the expression and function of *E. coli relA* in *M. smegmatis*.

Since ppGpp is known to be a growth regulator in various prokaryotes, the effect of ppGpp accumulation on the growth kinetics of *M. smegmatis* was studied. As expected, we observed a slow growth rate for the strain harboring pMatt2 in comparison to the one with pMatt1 (Fig. 4C). Although the time of entry into stationary phase for the two cultures was the same, the OD of the pMatt2 culture was lower than that of the pMatt1 culture. This observation has been reported previously in other organisms (34, 36). The observed growth kinetics, as expected, suggest that ppGpp is likely to have a mechanism of operation in mycobacteria similar to that in other prokaryotes. However, the overall change in growth kinetics in this case is quantitatively different (the generation time of pMatt1 and pMatt2 are 2.1 and 2.6 h, respectively) from the one noticed under carbon starvation (Fig. 1). This can be explained by the fact that global metabolism of the cell would be affected more by nutritional depletion than by accumulation of one regulatory factor. Furthermore, poor regulation of expression and subsequent outgrowth of suppressor variants would further reduce the effect of the regulatory molecule on the growth rate of the bacterial population.

In order to observe the morphological change as a consequence of ppGpp accumulation, the two strains (pMatt1 and pMatt2) were grown at 30°C in enriched medium to an OD of 0.4 and then were shifted to 44°C. Then heat-fixed smears were stained with carbol-fuchsin. The slides were observed under a bright-field microscope (×1,000 magnification). The strain carrying pMatt2 appeared as short cocci, in contrast to the pMatt1 strain, which appeared as long, thin, rod-shaped bacilli (Fig. 5). These microscopic observations again indicate that ppGpp plays a crucial role in the morphological changes in *M. smegmatis*.

Functional Characterization of *M. tuberculosis relA/spoT* in vivo (i) Overexpression of *M. tuberculosis relA/spoT* homologue in *M. smegmatis*. Upon analyzing the genome sequences, a putative ppGpp synthase has been identified in *M. tuberculosis* (7) and *Mycobacterium leprae* (11). Interestingly, the single gene identified has almost 50% homology to both *relA* and *spoT* (encoding ppGpp hydrolase) of *E. coli*. Hence, it is possible that mycobacteria have only one gene for both synthetic as well as hydrolytic activity of ppGpp. Furthermore, the *relA/spoT* homologues from the two species of mycobacteria are 97% identical in their amino acid sequence, which suggests that the gene is functionally conserved across the species in mycobacteria. Since the *relA/spoT* homologue of *M. smegmatis*

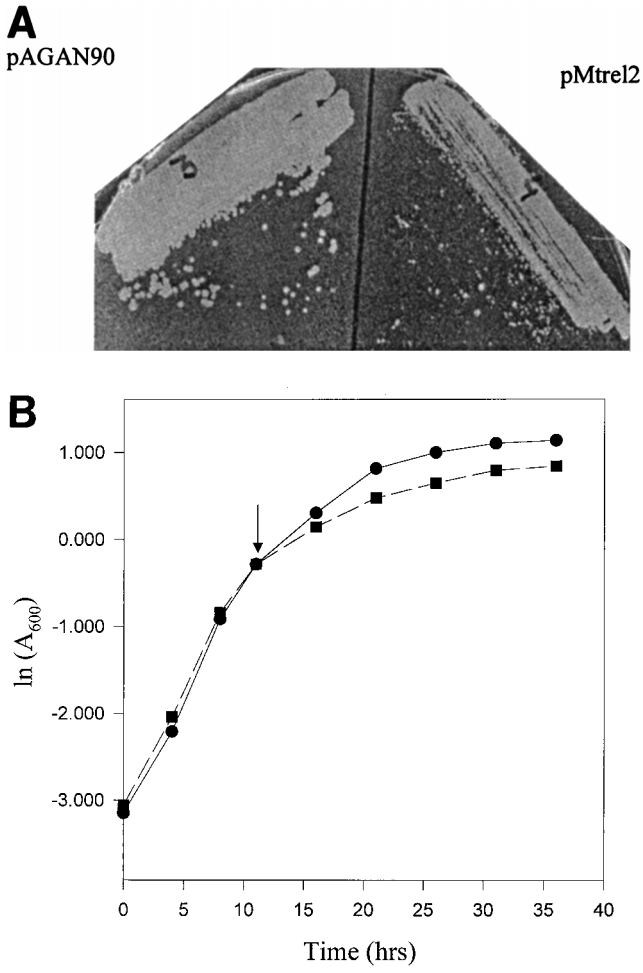

FIG. 8. Effect of ectopic expression of the *M. tuberculosis relA/spoT* on growth of *M. smegmatis*. (A) Reduction in colony size of the strain overexpressing *relA/spoT* (pMtrel2) as compared to the control (pAGAN90) when grown on MB7H9–2% succinate–2% acetamide. (B) Comparison of growth kinetics in liquid culture. The arrow indicates the time at which the inducer (2% acetamide) was added.

is not yet identified, we decided to characterize the function of *M. tuberculosis relA/spoT* using *M. smegmatis* as a surrogate host. In order to achieve this, another widely used mycobacterial promoter, $P_{amidase}$, was chosen. This promoter cassette is a 2.2-kb sequence with three ORFs and several consensus promoter sequences and is induced by addition of actamide to a medium having a poor carbon source (succinate) (29). However, the exact mechanism of induction is unknown. The *Eco*RV-*Kpn*I fragment (ORF Rv2583c, cosmid MTCY227) consisting of the *relA/spoT* ORF with its RBS was subcloned downstream of the 2.2-kb acetamide-inducible region of pAGAN90 (29), resulting in a transcriptional fusion of *relA/spoT* with an acetamide-inducible promoter.

The recombinant plasmid (pMtrel2) thus obtained was electroporated into *M. smegmatis*, and transformants were selected on MB7H9 agar containing kanamycin. The induction of the gene was seen when the strain bearing pMtrel2 was grown in MB7H9 broth containing 2% succinate till mid-log phase, with a subsequent addition of 2% acetamide. The Coomassie blue-stained gel used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed induced expression of one 89-kDa protein after 2 h of addition of the inducer (acetamide) (Fig. 6). The cellular content of the protein increased even after 20 h of induction, which indicates a long half-life of the protein.

(ii) Complementation of *E. coli relA* by *M. tuberculosis relA/spoT*. Since the 2.2-kb inducible region of pMtrel2 has appropriately placed *E. coli* consensus −35 and −10 sequences (21), it was assumed to be transcriptionally active in *E. coli*. Hence, the upstream regulatory region of pMtrel2 was thought to be sufficient for complementation in *E. coli*. Thus, the plasmid pMtrel2 containing *M. tuberculosis relA/spoT* was transformed into a *relA* strain of *E. coli* (MC4100). The transformant was checked for the $relA^+$ phenotype on a minimal medium plate with SMG. Since *relA* strains of *E. coli* are defective in derepression of the amino acid biosynthetic genes in amino acid-limiting medium, the cells are rendered sensitive to the presence of amino acids (through end product inhibition) in minimal medium (3). As can be seen in Fig. 7A, MC4100, which is sensitive to SMG, could form colonies on SMG plates when transformed with pMtrel2. This indicates that the gene coding for the 89-kDa protein can complement the RelA phenotype in *E. coli* and thus is a *relA* homologue.

(iii) ppGpp accumulation and its effect on cell growth upon induction of *relA/spoT* homologue. In the next experiment *relA/spoT* in pMtrel2 was induced in *M. smegmatis* as described above. The elevated intracellular level of ppGpp as a consequence of induction was observed (Fig. 7B). There was no detectable level of ppGpp either in the uninduced state of pMtrel2 or the empty vector, pAGAN90 (data not shown). The result indicates the ppGpp synthetic activity of the *relA/spoT* homologue. Although the synthesis of pppGpp by the same gene has been reported in an in vitro experiment (1), such a product could not be detected unambiguously in the formic acid extract because of comigration of some unknown spot. The growth kinetics of the strains having pMtrel2 and pAGAN90 were compared in culture as well as on plates (Fig. 8). The generation time of pMtrel2 was 4.1 h, compared to 2.5 h for pAGAN90, after induction. An enhanced reduction in growth rate compared to that in the pMatt1-pMatt2 system can be attributed to the controlled regulation of $P_{amidase}$. The slow growth of the strain having pMtrel2 upon induction is consistent with our previous observation that ppGpp downregulates the growth rate in *M. smegmatis*. The morphology of the two strains could not be compared because of severe clumping of the cells in the medium containing succinate and acetamide.

DISCUSSION

The experimental observation reported here indicates that morphological changes, from an elongated rod to a spherical coccus, in carbon-starved *M. smegmatis* may be due to elevated intracellular levels of ppGpp. It has been observed that other bacteria, like *S. enterica* serovar Typhimurium (13), *Vibrio vulnificus* (22), *Arthrobacteria crystallopoietes* (39), and *Pseudomonas putida* (14), undergo similar changes in low-nutrient medium, although the concomitant change in the ppGpp pool has not been established. It appears that such morphological change substitutes for a programmed differentiation (as seen in sporulating bacteria) in nondifferentiating bacteria. Although the molecular mechanism of bacterial size reduction is far from being clear, it is held that rapid cell division without an increase in cell mass results in the short spherical shape (19). Probably, the increase in cell number improves the strain survival during starvation. The fact that *M. smegmatis* undergoes a similar morphological change during the period of starvation shows a fundamentally common mechanism of bacterial survival under extreme growth conditions.

The involvement of ppGpp in cellular differentiation in *M. smegmatis* may provide an important clue towards understanding the survival of the organism. It can be perceived that *M. smegmatis* adopts a stringent physiology during starvation which results in a concomitant increase in the ppGpp pool. However, detailed studies on kinetics of macromolecular synthesis and degradation in a starved culture are required before a clear picture can be conceived.

The complementation study of the *relA*/*spoT* of *M. tuberculosis* in *E. coli* suggests a functionally conserved pathway in prokaryotes. However, the reason for the presence of a bifunctional (ppGpp synthase and hydrolase) gene in mycobacteria and related organisms, streptomyces, remains unanswered. Nevertheless, a possible role of stringent pathways in the developmental processes of even evolutionarily divergent species of bacteria cannot be ruled out.

The studies on starvation in mycobacteria bear relevance to the physiological state of latent tubercle bacilli. Because of similarities in morphology between starved cultures and natural persistors, the two can be argued to have the same metabolic activity. The change in bacterial shape as a consequence of ppGpp accumulation suggests an important role of the stringent factor in transformation of active bacilli into latent bacilli.

The role of ppGpp in pathogenesis appears to be interesting, based on a recent report showing that the nucleotide is a key switch in transformation of an avirulent to virulent form of *Legionella pneumophila* (15). Upon correlating the mechanisms of infection and natures of persistence between *L. pneumophila* and *M. tuberculosis*, we suggest an important role for ppGpp in the latency of the mycobacterium, and thus, studies on the stringent pathways would answer some important questions pertaining to the physiological transformation in this pathogen.

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