Regulation of *hmp* Gene Transcription in *Mycobacterium tuberculosis*: Effects of Oxygen Limitation and Nitrosative and Oxidative Stress

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The *Mycobacterium tuberculosis hmp* gene encodes a protein which is homologous to flavohemoglobin in *Escherichia coli*. Northern blotting analysis demonstrated that *hmp* transcription increased when a microaerophilic culture became oxygen limited as it entered stationary phase at 20 days. There was a fivefold increase of the *hmp* transcripts during early stationary phase compared with the value which was observed in the exponential growth phase. This induction of *hmp* transcription was not due to changes in the mRNA stability since the half-life of *hmp* mRNA was very short in a 20-day microaerophilic culture. No induction of *hmp* mRNA was observed during entry into stationary phase when the culture was continuously aerated. *hmp* transcription was induced after a short exposure of a late-exponential-phase culture to anaerobic conditions. These data indicate that oxygen limitation is the trigger for *hmp* gene transcription. In addition, when a microaerophilic culture entered into the stationary phase at 20 days, transcription of *hmp* increased to a small extent after exposure to *S*-nitrosoglutathione (a nitric oxide [NO] releaser) and sodium nitroprusside (an NO$^+$ donor) and decreased after exposure to paraquat (a superoxide generator) and H$_2$O$_2$. In log phase (4 days) and late stationary phase (40 days), the transcription of *hmp* was unaffected by nitrosative and oxidative stress. Three primer extension products were observed. The −10 region is 100% identical to that of promoter T3 in mycobacteria and shows a strong similarity to the −10 sequence of *hmp* and *rpoS* promoters in *E. coli*. These observations of *hmp* mRNA induction in response to O$_2$ limitation and nitrosative stress suggest that the *hmp* gene of *M. tuberculosis* may have a role in protection of the organism from NO killing under microaerophilic conditions.

Mycobacterium tuberculosis is the single most important infectious pathogen in the world and causes over 3 million deaths annually (23). *M. tuberculosis* persists in a dormant form in the body, by resisting the effects of the human immune response and chemotherapy. It has been reported that one-third of the human population may be latently infected with dormant tubercle bacilli which can reactivate, causing tuberculosis later in life (40). If it were possible to eradicate the latent organisms, the control of tuberculosis would be improved. An understanding of the molecular basis by which *M. tuberculosis* becomes dormant is a prerequisite for the identification of molecules in dormant bacteria which can be targeted by new drugs.

It is thought that low oxygen tension in the lesions may be responsible for *M. tuberculosis* entering a stationary-phase-like dormant state (5, 36, 39). If animals which have experimental tuberculosis are housed in low-oxygen-tension conditions, the level of the disease in the tissues is reduced (36); also the localization of adult pulmonary disease in subapical lesions may be explained by an O$_2$ tension higher than that in the lower parts of the lungs (39). An in vitro dormancy model (43, 45) in which tubercle bacilli settle through an oxygen-depletion gradient in unagitated broth cultures and undergo an orderly metabolic shiftdown with a change from oxygen-dependent metabolic pathways to the glyoxylate cycle (44) has been developed. *M. tuberculosis* is able to adapt to a nonreplicating persistent stage when the rate of dissolved O$_2$ is reduced in a controlled manner (46). This adaptation to microaerophilic and anaerobic conditions is associated with a marked increase

in nitrate reduction (47). It is not known which *M. tuberculosis* molecules respond to a change in oxygen tension. For other microorganisms, a hemoglobin-like protein which is controlled by changes in O$_2$ concentration has been described. It has been reported that an increase in cell density and a decrease in oxygen tension result in a 50-fold induction of *Vitreoscilla* single-domain hemoglobin (VtHB) (4). Overexpression of the *Vitreoscilla* globin (*vgb*) gene in *Escherichia coli* enhances growth under microaerophilic conditions, suggesting that the protein may have roles in O$_2$ storage or facilitation of O$_2$ transport (22). Transcriptional fusions of the *vgb* promoter in *E. coli* exhibit a five- to sevenfold induction in the reporter gene expression under microaerophilic conditions, suggesting that the *vgb* promoter is regulated by oxygen tension (11). Limitation of oxygen supply also causes a 20-fold increase in intracellular flavohemoprotein concentration in *Alcaligenes eutrophus* (35). Overexpression of VtHB protein in *E. coli* terminal oxidase mutants restored the enzyme activity of the cells, suggesting that VtHB protein may have oxidase activity (12). The expression of the *hmp* gene of *Bacillus subtilis*, encoding flavohemoglobin, is strongly induced in response to O$_2$ limitation and by nitrite (24). These observations indicate that *hmp* may be involved in anaerobic metabolism. The *hmp* expression in *E. coli* was not induced by low O$_2$ tension but was stimulated by nitrate, nitrite, and nitric oxide (NO), suggesting that *hmp* may participate in metabolism of nitrogen compounds (34). Unlike the single-domain VtHB protein, flavohemoglobin proteins usually have two domains. The N terminus contains the heme domain, which binds oxygen to form an oxygenated complex (21, 32), and the C-terminal domain is homologous to the ferredoxin NADP$^+$ reductase protein with potential binding sites for flavin adenine dinucleotide and NAD(P)H (1). It has been suggested that flavohemoglobin in *E. coli* might act as an

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oxygen sensor based on the observation that flavin reduction increases upon deoxygenation of the heme domain during oxygen limitation (33).

In addition to its putative roles in oxygen diffusion and metabolism, flavohemoglobin is also proposed to function in protection of microorganisms from oxidative and nitrosative stress. It has been reported that a strain of *Saccharomyces cerevisiae* in which the *hmp* gene is deleted becomes sensitive to oxidative stress (49). In *E. coli*, *hmp* expression was induced in the presence of paraquat (a superoxide generator) (28), NO, *S*-nitrosoglutathione (GSNO; an NO releaser), or sodium nitroprusside (SNP; an NO^+ donor) (29, 30). This induction is independent of the SoxRS regulon which is activated by superoxide-generating agents and NO (30). The *hmp* gene mutant is very sensitive to these nitrogen oxide species (30). It has been demonstrated that the *E. coli* flavohemoglobin may function as an NO dioxygenase which participates in *S*-nitrosothiol and NO metabolism and protects the bacterium against NO killing (16, 18). In *Salmonella typhimurium*, growth of *hmp* gene deletion mutants was not affected by paraquat and H_2O_2 but was sensitive to NO generators, acidified nitrite, and *S*-nitrosothiols (10). These observations suggest that flavohemoglobin of these microorganisms may play an important role in protection against oxidative and nitrosative stress.

The complete genome sequence of *M. tuberculosis* (7) has revealed an open reading frame which shows 30.8% identity in a 211-amino-acid overlap to *E. coli* *hmp*-encoded protein. The *hmp* gene of *M. tuberculosis* has been allocated in the category of 171 miscellaneous oxidoreductases and oxygenases in the genome sequence. Taking advantage of the complete *M. tuberculosis* genome sequence, we studied the transcription of the *M. tuberculosis* *hmp* gene under different conditions of oxygen supply and growth phases in order to investigate the possible link between *hmp* expression and differential gene expression associated with microaerophilic adaptation to stationary phase by using an in vitro microaerophilic model of dormancy (43, 45). We have recently reported that *M. tuberculosis* protein synthesis is shut down in this model (20). We also examined *hmp* transcription in response to various oxidative and nitrosative agents to investigate the possible roles of *hmp* in protection of *M. tuberculosis* from oxidative and nitrosative stress. Such data contribute to a better understanding of the molecular mechanisms of stationary-phase adaptation and survival in *M. tuberculosis*.

MATERIALS AND METHODS

Bacteria and culture. *M. tuberculosis* H37Rv was grown at 37°C in Middlebrook 7H9 medium containing 0.05% Tween 80 supplemented with 10% albumin dextrose complex (Difco Laboratories). Samples of a 10-day mid-log-phase culture were stored at −70°C. They were thawed and subcultivated once for 10 days before being inoculated 1:10 in fresh medium to form the experimental cultures. Microaerophilic growth was achieved by incubating 10-ml cultures in 28-ml screw-cap bottles without shaking for up to 60 days. Anaerobic cultures were obtained by incubating 3 ml of the cultures with loosened caps in a jar (GasPak 150 system; Becton Dickinson) in which H_2 and CO_2 were generated with GasPaks (Oxoid) and checked with anaerobic indicators (Oxoid). Aerated growth was obtained by incubating 10-ml cultures on a shaker (Vibrax; Kikalabortechnik) at 150 rpm; at 24-h intervals, the bottles were opened once in order to ensure sufficient oxygen supply. Estimation of viability as CFU was performed by the method described previously (20).

Estimation of oxygen consumption. Dissolved O_2 was measured in a sealed and undisturbed culture in a glass incubation chamber of the oxygen electrode system (Digital model 10) with the electrodes (a platinum working electrode and an Ag-AgCl reference electrode) mounted at the bottom of the chamber (Rank Brothers Ltd., Cambridge, United Kingdom). This method was used only to measure the oxygen consumption of the bacilli at the bottom of the container. Dissolved O_2 was also measured in a continuously disturbed culture which was stirred with a mini-magnetic bar at 100 rpm. At 1- to 2-day intervals, the incubation chamber was opened in order to ensure sufficient oxygen supply.
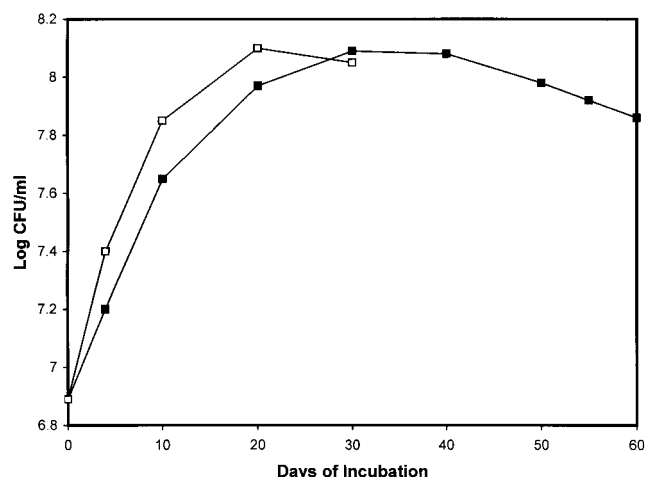

FIG. 1. Growth curves of *M. tuberculosis* H37Rv. The bacilli were grown in 7H9 medium containing 0.05% Tween 80 supplemented with 10% albumin dextrose complex without shaking and with shaking as described in Materials and Methods. Viability was estimated as CFU per milliliter at 0, 4, 10, 20, 30, 40, 50, 55, and 60 days of unagitated incubation (black squares; curve a) and at 0, 4, 10, and 30 days of agitated incubation (white squares; curve b). The values shown are the averages of three independent experiments.

DNA manipulations, sequencing, and analysis. DNA isolation, ethanol precipitation of DNA, electrophoresis of DNA in agarose, and transformations were performed by standard procedures (37). The TOPO TA cloning kit (Invitrogen) was used for cloning of PCR products. DNA for sequencing was isolated with a plasmid miniprep kit (Qiagen). Sequencing reactions were carried out on double-stranded plasmid DNA with the T7 Sequenase version 2.0 sequencing kit (U.S. Biochemical) in accordance with the manufacturer's instructions, based on the dideoxy chain termination method (38) with α-^{35}S-dATP (specific activity, >1,000 Ci/mmol; Amersham) as the radioactive label. Computer-aided analysis of the DNA sequence was performed by using the GCG sequence analysis software package (University of Wisconsin Biotechnology Center, Madison).

PCR amplification of DNA. PCR was used to generate a 620-bp sequence which began at 421 bp upstream and ended at 196 bp downstream of the TTG start codon of the *hmp* gene for sequencing the DNA ladder used in primer extension experiments. The primers were 5′-TGCACGCCGACGATTGA GC-3′ and 5′-TACGCTCGCTGGGCACGC-3′. The probes for detecting the *hmp* mRNA (EMBL-GenBank accession no. Z92774, coding sequence nucleotide [nt] 17664 to 18741), *cspA* mRNA (EMBL-GenBank accession no. Z95436, coding sequence nt 28425 to 28628), *ftsZ* mRNA (EMBL-GenBank accession no. Z95388, coding sequence nt 15919 to 17058), and 16S rRNA (EMBL-GenBank accession no. mtu16srn) were also prepared by PCR. PCR amplification was performed with 1 to 5 ng of *M. tuberculosis* chromosomal DNA in a final volume of 50 μl containing 1 μM (each) primers; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 1 U of *Taq* polymerase (Promega); and a buffer supplied with the enzyme. Amplification was carried out for 30 cycles as follows: denaturation for 1 min at 94°C, primer annealing for 2 min at 55°C, and primer extension at 72°C for 3 min. Primers which were designed to be in the coding regions of the transcripts were as follows: *hmp* (5′-TCACGGTCAAACGAACCGCC-3′ and 5′-GGGTTGTGGGGACGAAGTTG-3′; for position, see Fig. 5), *cspA* (5′-GA GAAGGGGTTCGGCTTTAT-3′, nt 28595 to 28576, and 5′-CTGGTTTTCTT CAAGGGTGC-3′, nt 28491 to 28510), *ftsZ* (5′-GTCGTGGGTATCGGTGGT GG-3′, nt 17022 to 17003, and 5′-ATCTCGTCCTTGGCGTCCTC-3′, nt 16802 to 16821), and 16S (5′-GCCTGGGAAACTGGGTCTAA-3′, nt 109 to 128, and 5′-TCTCCACCTACCGTCAATCC-3′, nt 427 to 446).

RNA extraction. Total RNA extraction from cultures was carried out by the method of Mangan et al. (25). After isopropanol precipitation, the RNA pellets were treated with RNase-free DNase I (Life Technologies), phenol and chloroform extracted, and reprecipitated. RNA concentration was determined spectrophotometrically at 260 nm.

Northern blotting analysis. Northern blotting analysis was performed by fractionation of RNA samples on a 1.2% agarose gel containing 6.5% formaldehyde, followed by transfer to a Hybond-N filter (Amersham) in 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (37). The same amount of total RNA (20 μg) was loaded into each well of the gel. Sizes were determined with an RNA ladder (Sigma) as the molecular size standard. The probe which was purified with QIAquick PCR purification kits (Qiagen) was labelled with [α-^{32}P]dCTP (specific activity, >3,000 Ci/mmol; Amersham) by the random priming method according to the instructions of the manufacturer (Amersham). Generally, 1×10^5 to 5×10^5 cpm/ml was used in hybridization for probes with
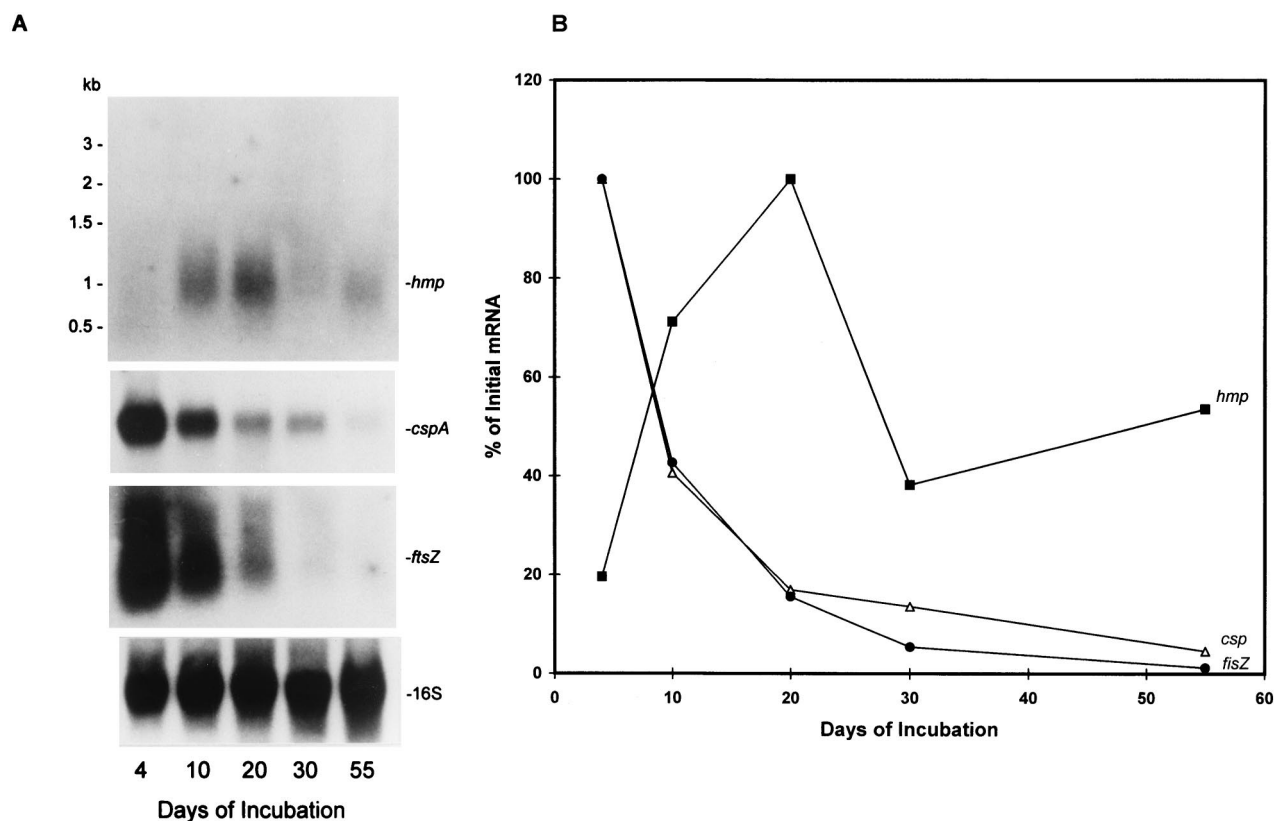
A

B

FIG. 2. Northern blotting analysis of steady-state levels of the *hmp* mRNA in *M. tuberculosis*. (A) RNA was extracted at 4, 10, 20, 30, and 55 days of microaerophilic incubation across growth curve a (Fig. 1) and analyzed by formaldehyde-agarose gel electrophoresis and Northern blotting as described in Materials and Methods. The filter was hybridized with an *hmp* gene-specific probe. The blot was stripped and reprobed with *cspA*-, *ftsZ* mRNA-, and 16S rRNA-specific probes. (B) Densitometric analysis of the autoradiographs and two other independent experiments showing the steady-state levels of the *hmp*, *cspA*, and *ftsZ* transcripts. The quantification was based on several exposures of different periods. The signal obtained from each band for each mRNA was divided by the corresponding signal of 16S rRNA. The corrected data of the bands for each mRNA were plotted against the days of incubation and expressed relative to maximal value.

a specific activity of more than 10^8 cpm/μg. After prehybridization for 3 h at 42°C in a buffer containing 5× Denhardt's solution, 5× SSC, 0.2% sodium dodecyl sulfate (SDS), 50% formamide, and 100 μg of salmon sperm DNA (Sigma) per ml, the filters were hybridized overnight at 42°C with ^{32}P-labelled probes in the same buffer and washed at high stringency (6× SSC–1% SDS, 1× SSC–1% SDS, and 0.1× SSC–1% SDS at 68°C for 45 min, respectively). The same filters were stripped and reprobed for 16S rRNA to verify equal loading of RNA. The filters were exposed to X-ray films. The films, exposed for various periods, were scanned with a high-resolution laser Personal Densitometer SI (Molecular Dynamics) linked to ImageQuant software (Molecular Dynamics).

Chemical half-life determination. Total RNA was isolated from an early-stationary-phase (20 days) microaerophilic culture at selected intervals after transcription initiation was inhibited by the addition of 100 μg of rifampin (Sigma) per ml, and the half-life was determined by Northern blotting analysis. The incorporation of [^{3}H]uridine (activity, 31 to 56 Ci/mmol; Amersham) into trichloroacetic-acid-precipitable RNA was rapidly reduced to 2% of that of the drug-free control (data not shown) after addition of 100 μg of rifampin per ml, showing that transcription initiation was blocked with this concentration of rifampin.

Analysis of RNA accumulation under stress conditions. A series of 10-ml cultures were used for the determination of stress responses. For oxidative stress, H_2O_2 and paraquat were added to the cultures at the final concentrations of 10 mM and 200 μM, respectively, for 30 min. For nitrosative stress, GSNO and SNP were added to the cultures at the final concentrations of 1 and 10 mM, respectively, for 1 h. RNA was extracted after exposure of the cells to these stress conditions and analyzed by Northern blotting.

Primer extension. The synthetic oligonucleotide PE1 (5′-GCCGAGTGGCT CGTCTCCAA-3′, 12 to 31 nt downstream of *hmp* gene TTG start codon) and PE2 (5′-TCGTCGACGAAACCGACG-3′, 62 to 79 nt downstream of *hmp* gene TTG start codon) were 5′ end labelled with [γ-^{32}P]ATP (specific activity, >3,000 Ci/mmol; Amersham) by using Ready-To-Go T4 polynucleotide kinase (Pharmacia Biotech). Prior to primer extension, the total RNA was analyzed by Northern blotting and hybridized with the 16S rRNA-specific probe in order to verify that equal amounts of total RNA were used for each time point. Total

RNAs (40 μg) from different growth phases were annealed with 1 μl of 5′-end-labelled primer (approximately 10^5 cpm) in 5× reverse transcriptase buffer (Life Technologies) at 72°C for 20 min and then slowly cooled to room temperature in 30 min. Primer extension was performed in the same solution for 1 h at 42°C containing 500 μM (each) dATP, dCTP, dGTP, and dTTP; 40 U of RNasin (Promega); 5 mM dithiothreitol; and 200 U of Superscript II reverse transcriptase (Life Technologies). The primer extension products were precipitated with ethanol and sodium acetate at −70°C, washed with 70% ethanol, and dried. The pellets were resuspended in an appropriate amount of formamide dye solution (U.S. Biochemical) and then separated on a 6% polyacrylamide sequencing gel containing 8 M urea adjacent to a DNA sequence generated with the same primer.

Nucleotide sequence accession number. The nucleotide sequence accession number for the *M. tuberculosis hmp* gene is EMBL-GenBank Z92774 (coding sequence nt 17664 to 18741); the gene name is Rv3571 (7).

RESULTS

***hmp* gene transcription in different growth phases.** In order to determine if *hmp* gene transcription was dependent on the growth phase of a culture, RNA was isolated from bacilli taken at different growth phases under unagitated microaerophilic conditions across the growth curve (Fig. 1, growth curve a) and was analyzed by Northern blotting. In our model (Fig. 1, growth curve a), entry into stationary phase, defined as slowing of log-phase growth, began at about 10 to 20 days and ended at 30 to 40 days when the bacilli entered the stationary phase. Oxygen concentration, which was measured at the bottom of the culture, decreased from 20% (air-saturated 7H9 medium) to 0.1% during the first 24 h of incubation and remained at
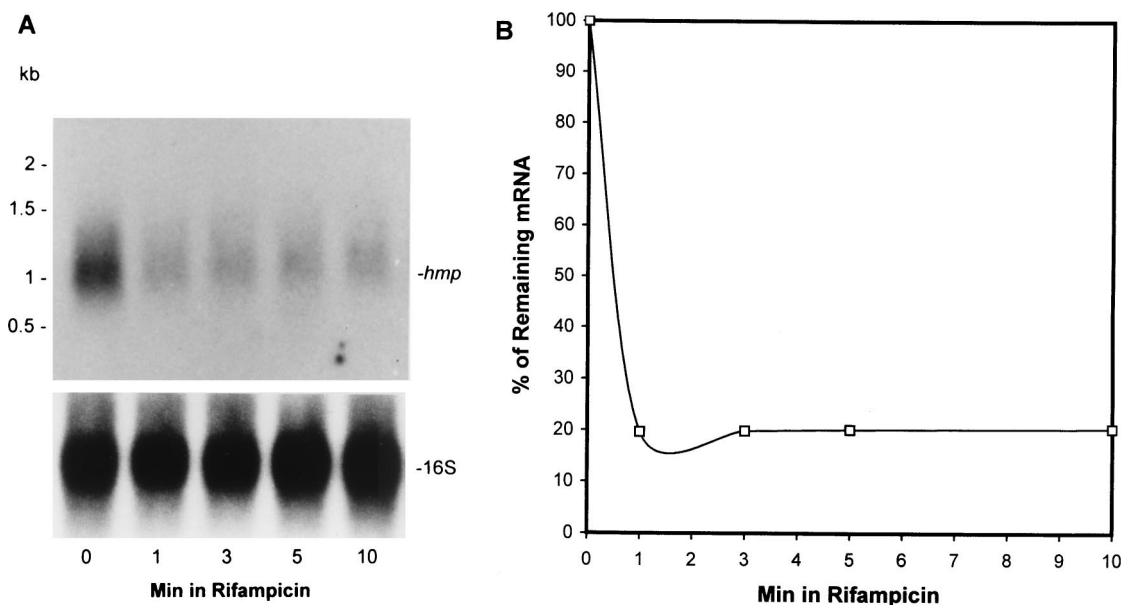
FIG. 3. Northern blot analysis of decay of *hmp* mRNA in 20-day-old stationary-phase bacilli. (A) Total RNA was extracted from the bacilli at 0, 1, 3, 5, and 10 min after addition of 100 μg of rifampin per ml. (B) Densitometric analysis of decay of *hmp* mRNA. The quantification is based on several exposures of the autoradiographs (A) and two others from independent experiments for different periods. The signals of the bands were plotted against the times of the RNA isolation and expressed as the percentages of the initial values. The half-life calculated from the blots was 0.8 ± 0.05 min.

<0.1% for the rest of the incubation period. This indicates that in these cultures the oxygen gradient is established within 24 h, after which a low concentration of oxygen is maintained in the bottom of the culture and a higher but gradually decreasing oxygen concentration is maintained in the top of the culture (43, 44). In log-phase-growth cultures, the majority of the bacilli are dispersed throughout the culture, but in unagitated cultures, they gradually settle to the bottom of the container over several days where they enter the stationary growth phase. As shown in Fig. 2A (top panel), the *hmp* RNA was weakly expressed during exponential growth (4 days) when there was an abundance of oxygen in the top layer of the medium. As the culture entered the late exponential growth period at 10 days, when oxygen tension began to decrease as a result of bacterial consumption, *hmp* transcription increased and reached its maximum level at early stationary phase (20 days). After this, the transcript level decreased while the bacilli adapted to the late stationary phase. The length of the *hmp* transcript is approximately 1.1 kb, and this matches the prediction of the *hmp* mRNA length based on DNA sequence and primer extension analysis (see below). The blots were stripped and reprobed to detect 16S rRNA, *cspA* mRNA, and *ftsZ* mRNA. The purpose of examining *cspA* and *ftsZ* mRNA in these samples was to compare the transcription of other genes with that of *hmp*. *cspA* encodes cold shock protein A, which has 73.4% amino acid identity to the cold shock protein of *Arthrobacter globiformis* (3). *ftsZ* encodes a cell division protein having 77.3% amino acid identity to the *ftsZ* gene product of *Streptomyces coelicolor* (26). As shown in Fig. 2A, the levels of *cspA* and *ftsZ* mRNA were maximal at log-phase growth (4 days) and then gradually decreased as the bacilli reached stationary phase, in agreement with our previous finding that total protein synthesis showed substantial reduction in the progress to stationary phase (20). The level of 16S rRNA (Fig. 2A, bottom panel) was relatively constant throughout different growth phases, indicating that equal amounts of total RNA were loaded into each well of the gel. Densitometric analysis of the autoradiographs shown in Fig. 2A and others from two independent experi-

ments revealed that there was a 5- ± 0.02-fold increase of the *hmp* transcript in early stationary phase relative to the values observed in the early exponential phase. In contrast, there were 22- ± 0.07-fold and 23- ± 0.07-fold decreases of the *cspA* mRNA and *ftsZ* mRNA, respectively, in late stationary phase compared with the value measured in log phase (Fig. 2B).

Determination of the *hmp* mRNA half-life in a microaerophilic culture. Since the steady-state level of any mRNA species is determined by the rate of transcriptional initiation and the rate of decay, we determined the *hmp* mRNA half-life in a 20-day stationary-phase culture after transcription initiation was inhibited with 100 μg of rifampin per ml. The autoradiograph shown in Fig. 3A (top panel) revealed that the *hmp* mRNA was very unstable, with a half-life of less than 1 min determined by densitometric analysis (Fig. 3B). This indicates that the high level of *hmp* mRNA which is present at 20 days is due to an increase in transcriptional initiation and not due to enhanced stability of the mRNA. The blot was stripped and reprobed to detect 16S rRNA (Fig. 3A, bottom panel) in order to ensure that an equal amount of total RNA was loaded into each lane. Determination of the *hmp* half-life in log-phase and late-stationary-phase cultures was not feasible since the steady-state levels of the *hmp* mRNA in those cultures were very low (Fig. 2A) and the decay of the mRNA after treatment with rifampin was below the level of detection.

***hmp* gene transcription under aerobic and anaerobic conditions.** We then posed the question whether *hmp* induction, during entry into the stationary phase, was due either to the decrease in oxygen tension caused by bacterial consumption or to growth rate changes independent of O_2 level. We thus measured the transcription of the *hmp* gene under aerobic conditions. Total RNA was prepared from samples taken after 4, 10, and 20 days of aerobic incubation across the growth curve (Fig. 1, growth curve b) and subjected to Northern blotting analysis. O_2 concentration measured at the bottom of the culture with continuous stirring decreased from 20 to 18% over 30 days of the incubation, showing no significant change. As shown in Fig. 4A (top panel), *hmp* was expressed at low levels throughout

A

-hmp

-cspA

-16S

4   10   20

Days under Aerobic Conditions

B

-hmp

-cspA

-16S

0   1   4
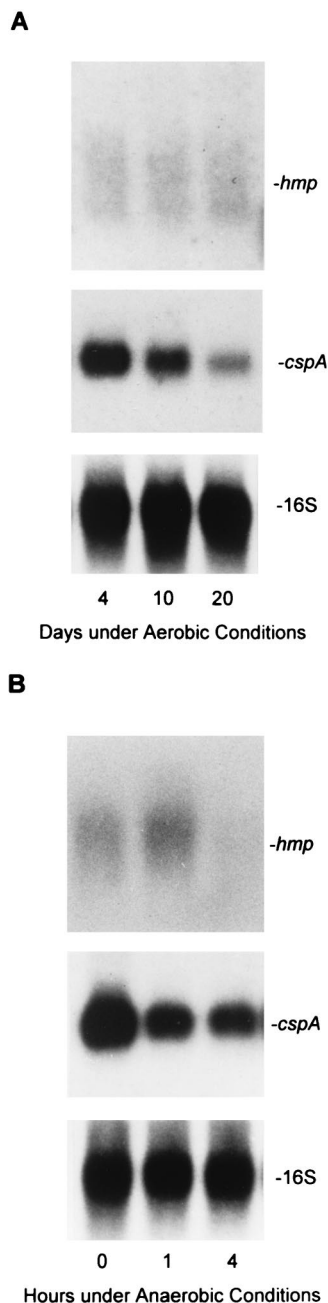
Hours under Anaerobic Conditions

FIG. 4. Effects of aerobic and anaerobic conditions on transcription of the *hmp* gene. (A) RNA was extracted at 4, 10, and 20 days of aerobic incubation (Fig. 1, growth curve b) and analyzed by Northern blotting. The filter was hybridized to detect *hmp* mRNA (upper panel) and then reprobed to detect *cspA* mRNA (middle panel) and 16S rRNA (lower panel) after being stripped. (B) RNA was extracted from a mid-log-phase culture (7 days) (Fig. 1, growth curve b) after exposure to anaerobic conditions for 0, 1, and 4 h and subjected to Northern blotting analysis. Hybridization of *hmp* mRNA (upper panel) and rehybridization of *cspA* mRNA (middle panel) and 16S rRNA (lower panel) were performed as described for panel A. These results have been independently confirmed in two additional experiments.

different growth phases, showing no induction of the mRNA when the bacilli entered the stationary phase with respect to continuous oxygen availability. The blot was stripped and rehybridized to detect *cspA*. The steady-state level of *cspA* mRNA exhibited the similar pattern as that in microaerophilic

growth, in which an approximately 6.7- $\pm$ 0.04-fold decrease was observed in stationary phase compared to log-phase growth (Fig. 4A, middle panel). The blot was stripped and reprobed with the 16S rRNA probe and showed that an equal amount of total RNA was loaded in each lane (Fig. 4A, bottom panel).

To further investigate the effects of growth phase and oxygen tension on the *hmp* expression, we took a mid-log-phase microaerophilic culture (7 days) and exposed it to anaerobic conditions to examine if *hmp* is up-regulated by low oxygen in mid-log-phase bacilli rather than the early-stationary-phase bacilli. As shown in Fig. 4B, after exposure of the culture to anaerobiosis for 1 h, the *hmp* mRNA increased approximately 1.45- $\pm$ 0.05-fold and decreased rapidly after 4 h of anaerobic incubation (top panel). Exposure to anaerobiosis did not kill the bacilli since CFU counts remained constant after 24 h of anaerobic incubation. In contrast, anaerobic incubation for 1 and 4 h resulted in 2.2- $\pm$ 0.05-fold and 2.5- $\pm$ 0.05-fold reduction of *cspA* mRNA, respectively (Fig. 4B, middle panel). The equal loading of total RNA was confirmed by the same intensity of the 16S rRNA bands shown in Fig. 4B, bottom panel. These observations suggest that oxygen limitation rather than growth rate change is the real trigger for *hmp* induction.

***hmp* gene transcription under oxidative and nitrosative stress conditions.** In order to determine if the *hmp* expression was affected by oxidative and nitrosative stress, we exposed a 20-day microaerophilic culture to H_2O_2, paraquat, GSNO, and SNP and analyzed the transcription of *hmp* by Northern blotting. As shown in Fig. 5A (top panel) and 5B, compared with the steady-state level of the mRNA (control), transcription of *hmp* was increased 1.4- $\pm$ 0.01-fold after exposure to SNP and 1.6- $\pm$ 0.04-fold after exposure to GSNO. The level of *hmp* mRNA decreased after exposure to hydrogen peroxide and paraquat. The blot was stripped and reprobed to detect 16S rRNA (Fig. 5A, bottom panel) in order to ensure that an equal amount of total RNA was loaded into each lane. The same experiments were also performed with log-phase (4-day) and late-stationary-phase (40-day) cultures. No changes in the level of *hmp* mRNA were observed in response to any of the stress conditions (data not shown). Viability of the bacilli was assessed by CFU counts after exposure of the organisms to the stress conditions described above. The CFU counts were unaltered by any of these stress conditions.

Primer extension analysis. Total RNA was harvested at various times from the microaerophilic growth cultures (Fig. 1, growth curve a) and was subjected to primer extension analysis by using the oligonucleotide primer PE1 in order to identify the transcription start sites for the *hmp* mRNA. As shown in Fig. 6, three primer extension products of 58, 55, and 51 nt which correspond to G, A, and C positions 25, 22, and 18 nt (Fig. 7) upstream of the TTG start codon were found. This identifies the potential 5′ end of *hmp* mRNA of about 1.1 kb in size. This result was independently confirmed by using the second primer, PE2 (Materials and Methods). As shown in Fig. 7, when the sequence centered around −10 and −35 regions upstream from the transcription start sites was examined for promoter-like sequences, we found that the −10 region (TAACAT) contains a highly conserved sequence identical to the mycobacterial promoter T3 which is one of the promoters randomly selected by a shuttle vector (2) and shows a clear similarity to the −10 region (TAAGAT) of the *E. coli hmp* promoter (27) and the −10 sequence (TATCAT) of the *E. coli katF* (σ^S gene) promoter (31). The sequence in the −35 region, assuming an optimal spacing of 17 bp between −10 and −35, has a 3- of 6-nt match to the *E. coli* −35 consensus sequence (TTGACA) but has no similarity to that of mycobacterial pro-
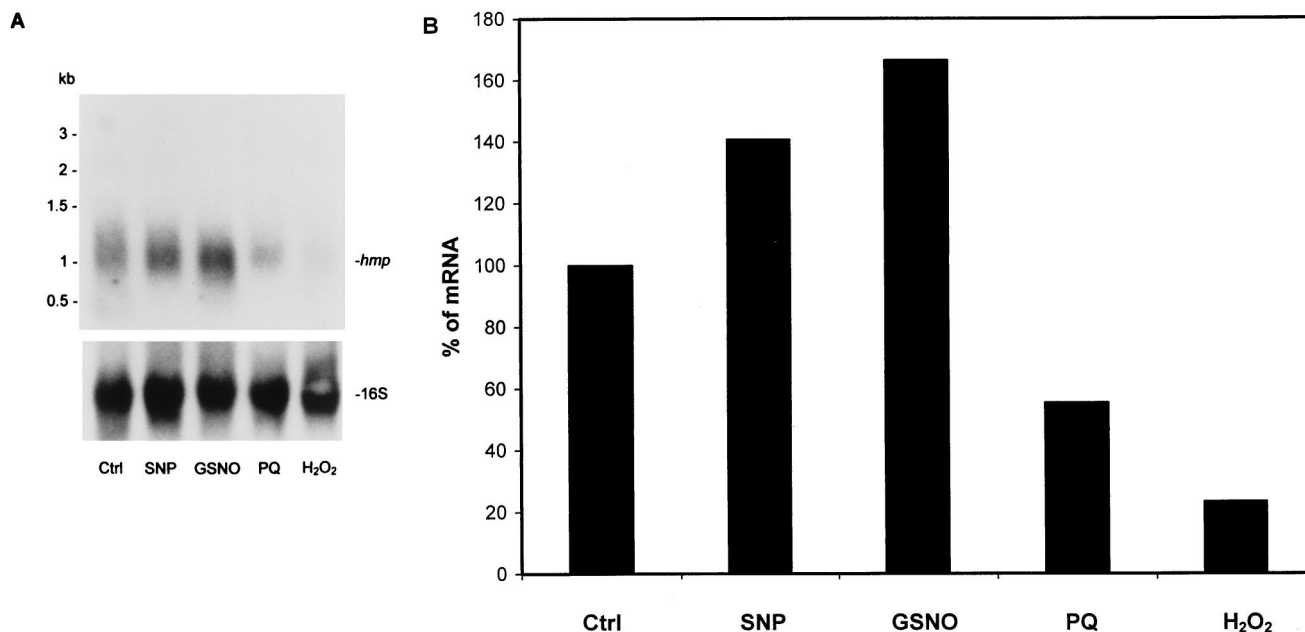
A

B

FIG. 5. Northern blot analysis of the *hmp* mRNA level in response to nitrosative and oxidative stresses. RNA was extracted from a 20-day microaerophilic culture after exposure to different stress conditions described in Materials and Methods and subjected to Northern blot analysis. (A) Northern blot hybridization of *hmp* mRNA. The same blots were stripped and hybridized with the 16S rRNA-specific probe. (B) Densitometric analysis of *hmp* mRNA level in response to stresses. The values shown are the averages of two independent experiments. Ctrl, control; PQ, paraquat.

moters, which was consistent with previous findings (2). A highly conserved Shine-Dalgarno sequence is centered 8 bp upstream of the TTG initiation codon. The upstream noncoding region was amplified by PCR, cloned, and sequenced and was identical to the DNA sequence (GenBank accession no. Z92774) from the *M. tuberculosis* chromosome.

Primer extension analysis was also used to examine the temporal patterns of *hmp* gene transcription (Fig. 6). The signals were much stronger during entry into the stationary phase. Densitometric analysis revealed that the temporal patterns of the three bands were very similar and closely matched the patterns in Northern blotting.

DISCUSSION

We have shown that oxygen tension modulates *M. tuberculosis hmp* gene transcription. Northern blotting analysis revealed that transcription of the *hmp* gene increased when a microaerophilic culture became oxygen limited as it reached stationary phase. This was not due to the changes in the mRNA stability since the *hmp* mRNA was very unstable in a 20-day stationary-phase culture. This increase in *hmp* transcription was not observed throughout different growth phases when the cultures were continuously aerated, indicating that oxygen abundance inhibits *hmp* expression. Furthermore, *hmp* transcription was significantly induced after a short exposure of an exponential-growth-phase culture to anaerobic conditions, showing that an increase in *hmp* expression is associated with a decrease in oxygen tension. Our previous studies have demonstrated that *M. tuberculosis*, under anaerobic conditions, rapidly reduces or completely switches off protein synthesis in order to achieve a shutdown of cellular metabolic activity (20). *hmp* mRNA disappears quickly under further anaerobic incubation. A possible explanation for this might be the metabolic shutdown which is seen during anaerobiosis. The transient increase in the level of *hmp* mRNA during entry into stationary

growth phase when O_2 became limiting was in contrast to the mRNA levels of other genes such as *cspA* and *ftsZ* mRNAs, whose transcription decreased during the same period. These observations suggest that oxygen limitation triggers *hmp* induction. In addition, we have observed that transcription of the *hmp* gene in early-stationary-phase cultures was slightly increased in response to GSNO and SNP, suggesting that the *hmp* gene product may be involved in the protection of the organisms from nitrosative stress.

The primer extension analysis reveals three termination points which may be representative of 5′ ends of the mRNA. However, these three signals do not necessarily mean that there are three transcription start sites, since the two shorter products may result from either incomplete extension of the primer or the degradation or processing of the mRNA species. It is possible that the longest extension product reveals the potential *hmp* transcription start site. Further experiments with S1 nuclease mapping will help to resolve these possibilities. The primer extension results together with Northern blotting data suggest that the unit of transcription might be monocistronic, which is consistent with the findings for *E. coli* (27). The *hmp* gene also contains a potential ribosome-binding site which is just upstream of the TTG start codon and is correctly located to initiate the translation of an open reading frame. Hence, the most likely translational start site is the TTG codon. In *M. tuberculosis*, TTG start codons have been predicted for approximately 4% of the coding regions (7, 31a). The putative promoter sequences of the *M. tuberculosis hmp* gene show a similarity to that of the *E. coli hmp* gene. In *E. coli*, induction of *hmp* in stationary phase is dependent on σ^S (27); also, σ^S is the dominant regulator of *hmp* expression in the presence of paraquat during stationary phase (28). It is not known which σ factor controls the expression of the *hmp* gene. Recently, it has been demonstrated that the transcription of the *M. tuberculosis sigB* gene is induced during transition from log phase to stationary phase and under stress conditions,
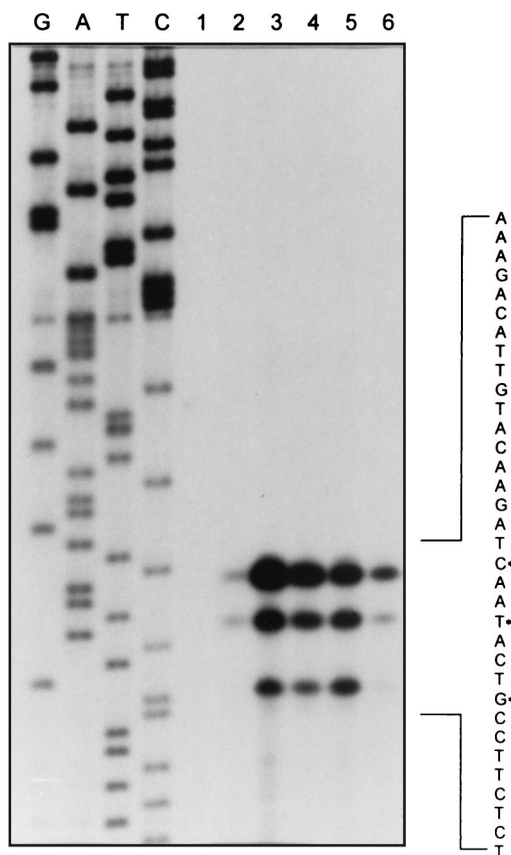
FIG. 6. Determination of the *hmp* transcription initiation site. Primer extension analysis was performed as described in Materials and Methods with total RNA prepared from cultures at different growth phases. Lanes G, A, T, and C contained sequence reactions generated with the same primer. Lanes 2, 3, 4, 5, and 6 indicate the primer extension products with the RNA isolated from the bacilli after 4, 15, 20, 25, and 30 days of unagitated incubation (Fig. 1, growth curve a), respectively. A reaction without RNA is shown in lane 1. Primer extension points are marked by asterisks in the DNA sequence shown on the right, which is the nontranscribed strand and is the complement of the sequence that is readable from the sequencing ladder.

```
-121  TTACTTTTGC CTCTCCGGGT TGCGAACTTA GAGAAAGACT AGAACACGTT
                                                 -35
 -71  CCGATTTGTG TCGAGCTAGG TATTCCTGCG GCAGGTAGCG ATACCAAATG
              -10              *  *  *         SD
 -21  GGTTTTCTGT AACATGTTCT AGTTATGACG GAAGAGAGGA CGGGTCTTGA
  30  CCGAGGCAAT TGGAGACGAG CCACTCGGCG ACCACGTCCT TGAACTGCAG
  80  ATCGCCGAGG TCGTCGACGA AACCGACGAG GCGCGATCGC TGGTCTTCGC
 130  GGTGCCCGAC GGATCGGACG ACCCGGAGAT CCCCCCTCGG CGCCTGCGTT
 180  ACGCCCCCGG CCAATTCTTG ACGCTGCGCG TGCCCAGCGA GCGTACCGGT
 230  TCGGTGGCGC GCTGCTACTC GTTGTGCAGT TCGCCCTACA CCGACGACGC
 280  CTTGGCGGTC ACGGTCAAAC GAACCGCCGA CGGGTACGCC TCCAACTGGT
 330  TGTGCGATCA CGCGCAGGTG GGCATGCGCA TCCACGTGCT GGCCCCGTCG
 380  GGCAACTTCG TCCCCACAAC CCTCGACGCC GATTTCCTCC TGCTGGCAGC
 430  GGGTAGCGGC ATCACCCCGA TCATGTCGAT CTGCAAATCG GCGCTTGCCG
 480  AGGGCGGTGG ACAGGTGACG CTGCTCTACG CCAACCGCGA CGACCGCTCG
```

FIG. 7. DNA sequence of the *hmp* gene regulatory region. Shown are the partial region encoding *hmp* and a 146-bp upstream sequence. The three primer extension points which correlated with nucleotides G, A, and C are indicated by asterisks. The putative promoter regions are highlighted by boxes and labelled −10 and −35. The putative ribosome-binding site is marked with a double underline and is labelled SD. The start codon is in boldface. The primers used for generating the Northern blotting probe are underlined.

which suggests that the *sigB* gene may encode an alternative sigma factor (19).

Hemoglobin-like genes are present in many microorganisms including *E. coli* (1, 21, 41), *Vitreoscilla* sp. (4, 11, 12, 22, 42), *A. eutrophus* (8, 35), *Erwinia chrysanthemi* (13), *B. subtilis* (24), *Rhizobium meliloti* (17), *S. typhimurium* (10), and *S. cerevisiae* (9, 49). It is still not clear what role the proteins play in the microorganisms, although several possible functions have been proposed, including oxygen transport and storage, oxidase and reductase activities, and oxygen sensing (reviewed in reference 32). The *hmp* gene may also be involved in anaerobic metabolism. In *B. subtilis*, *hmp* gene induction under anaerobic conditions is dependent on ResDE (two-component signal transduction proteins), FNR (anaerobic regulator), and NarGHJI (respiratory nitrate reductases) (24). In *E. coli*, *hmp* expression is negatively regulated by FNR under anaerobic conditions, is induced by nitrite and NO, and may participate in anaerobic metabolism of nitrogen compounds (34). No binding sites identical to *E. coli* FNR protein were found in the region of the *hmp* promoter in *M. tuberculosis*, indicating a possible difference in regulation mechanisms or functions of *hmp* between the two species. It is not known what role the *M. tuberculosis hmp* gene plays in response to O$_2$ limitation, and its involve-

ment in anaerobic metabolism requires further investigation. Recently, a body of evidence has arisen from the discovery that the bacterial *hmp* gene might function in response to nitrosative or oxidative stress (10, 16, 18, 28–30). A defined *hmp* gene mutant of *E. coli* is hypersensitive to nitrosating agents and NO-related species (30). Hmp protein may act as an NO dioxygenase which converts NO to NO$_3^-$ (16, 18). The *hmp* gene deletion mutant of *S. typhimurium* showed an increasing sensitivity to acidified nitrite and *S*-nitrosathiols, but the growth of the mutant was not inhibited by oxidative stress (10). These observations strongly suggest that the *hmp* gene plays an important role in protection of bacteria from NO attack. We found that the *hmp* gene in *M. tuberculosis* was induced to a small extent by nitrosating agents under microaerophilic conditions. *M. tuberculosis* is capable of replicating and persisting within mononuclear phagocytic cells. Activated macrophages generate reactive nitrogen intermediates (RNI) including NO, NO$_2^-$, and NO$_3^-$, which play an important role in controlling the bacteria in the host (6, 14). The ability of *M. tuberculosis* to survive RNI attack involves an array of proteins which are synthesized in response to this stress. The 16-kDa α-crystalline-like protein, which is a stationary-phase-associated protein (48), is induced under RNI stress (15). The induction of *hmp* mRNA in response to these NO generators indicates that the Hmp protein might have a role in the protection of *M. tuberculosis* from nitrosative stress when O$_2$ tension decreases, which might help the organism to persist in inflammatory and necrotic lesions of the human host. The *hmp* mRNA level was reduced by oxidative stress, which was consistent with the finding for *S. typhimurium* (10), suggesting that the regulation of the response to NO is different from that of the response to oxidative stress in *M. tuberculosis* and *S. typhimurium*. Gardner et al. suggested that the induction of (flavo)hemoglobins under microaerophilic conditions might be beneficial for bacteria to survive NO killing since the cellular level of NO dioxygenase increased when O$_2$ became limiting (16). It is not known if this is the case in *M. tuberculosis*. Future work will aim to construct an *hmp* gene deletion mutant of *M. tuberculosis* which will help to elucidate the role that *hmp* may play in response to oxygen limitation and nitrosative stress.

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