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Elements of Signal Transduction in *Mycobacterium tuberculosis*: In Vitro Phosphorylation and In Vivo Expression of the Response Regulator MtrA

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A putative two-component system, *mtrA-mtrB*, was isolated from *M. tuberculosis* H37Rv by using *phoB* from *Pseudomonas aeruginosa* as a hybridization probe. The predicted gene product of *mtrA* displayed high similarity with typical response regulators, including AfsQ1, PhoB, PhoP, and OmpR. The predicted gene product of *mtrB* displayed similarities with the histidine protein kinases AfsQ2, PhoR, and EnvZ and other members of this class of proteins. Expression analysis in the T7 system showed that *mtrA* encoded a polypeptide with an apparent molecular mass of 30 kDa. MtrA was overproduced, purified, and demonstrated to participate in typical phosphotransfer reactions using a heterologous histidine protein kinase, CheA, as a phosphoryl group donor. *Mycobacterium bovis* BCG, harboring an *mtrA-gfp* (green fluorescent protein cDNA) transcriptional fusion, was used to monitor *mtrA* expression in infected J774 monolayers. Flow cytometric and fluorescence microscopic analyses indicated that the *mtrA* promoter was activated upon entry and incubation in J774 macrophages. In contrast, the *hsp60-gfp* fusion displayed no change in expression under the growth conditions tested. These results suggest a potential role for *mtrA* in adaptation of the *M. tuberculosis* complex organisms to environmental changes which may include intracellular conditions.

Mycobacterium tuberculosis is notorious for both its slow growth and global importance as a human pathogen (4). Although there is a relatively safe vaccine, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), against tuberculosis, its effectiveness appears to be variable (29, 32). Efforts to develop new vaccines and therapeutic approaches are hampered by the paucity of information regarding potential virulence factors of *M. tuberculosis* (31). Improved knowledge and identification of genes and functions critical for host-pathogen interactions that render this organism such a successful human pathogen are needed in order to refine the existing treatments or provide novel targets for intervention. In this context, and in the area of *M. tuberculosis* fundamental biology, the perception of this bacterium as an inert participant in pathogenic processes continues to be challenged by the latest advances in molecular genetic analyses. However, in some instances the views of *M. tuberculosis* as a static organism have received affirmation. For example, it has been recently reported that at least in one case, *M. tuberculosis* has a nonfunctional regulator of oxidative stress, *oxyR* (11, 35), whose homologs in other organisms are critical to their adjustments to endogenous and exogenous reactive oxygen intermediates (13). The *oxyR* gene is inactivated by multiple lesions in all strains of *M. tuberculosis* tested and in all members of the *M. tuberculosis* complex, representing a phenomenon that has been linked to the exquisite sensitivity of this organism to isonicotinic acid hydrazide (11). This finding appears to support the notion of preset expression levels for at least some subsets of genes in *M. tuberculosis.*

In continuation of our investigations of the regulatory makeup of *M. tuberculosis*, the current study was aimed at testing this organism for the presence of signal transduction elements from the superfamily of bacterial two-component phosphotransfer systems. Bacteria show a wide range in the abundance of two-component systems; for example, while it is estimated that enteric bacteria may encode close to 50 response regulators (24, 37), only four such systems have been identified in *Haemophilus influenzae* (14), while another highly specialized pathogen, *Mycoplasma genitalium*, has none (15). The latter example further underscores the importance of determining whether *M. tuberculosis*, a highly and possibly terminally adapted human pathogen, has elements of signal transduction.

To investigate whether *M. tuberculosis* utilizes these types of environmental sensors, we isolated and characterized a two-component system from *M. tuberculosis* H37Rv. Furthermore, using a new in vivo expression detection technology based on green fluorescent protein (GFP) (5), recently adapted for use in mycobacteria (12, 20), we found that the *mtrA* gene is induced upon infection of macrophages.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Mycobacterial species and strains used were *M. tuberculosis* H37Rv (ATCC 27294), *M. bovis* BCG (Pasteur; ATCC 27291), and *Mycobacterium smegmatis* mc^{2}155 (36). The *phoB* gene from *Pseudomonas aeruginosa* (1) (plasmid pPHOB3) was from A. Lazdunski. The cosmid clone T410 was from S. Cole. Plasmids p*mtrA-gfp* and p*hsp60-gfp* have been previously described (12). Mycobacteria were grown in Middlebrook 7H9 broth or 7H10 agar (Difco Laboratories) supplemented with 0.5% glycerol, 10% ADC (albumin-dextrose complex) enrichment without catalase, and 0.05% Tween 80 (Sigma) at 37°C in the presence of 5% CO_2 as described by Jacobs et al. (19). When required, media were supplemented with kanamycin (25 μg/ml).

Cell culture and media. Murine BALB/c macrophage cell line J774A (ATCC TIB-67) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 200 mM L-glutamine, 5% heat-inactivated fetal bovine serum (FBS; HyClone), and no antibiotic supplements. Macrophage monolayers were maintained at 37°C in humidified air containing 5% CO_2 before and after infection as described previously (12).

Nucleic acid manipulations, recombinant DNA methods, and analyses. All

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DNA manipulations were carried out as previously described (2). Dideoxyribonucleotide chain termination DNA sequencing was carried out by using Sequenase (version 2.0; U.S. Biochemical), double-stranded templates, and custom-made oligonucleotides as sequencing primers spanning the 2.5-kb region reported in this study. Regions with difficult compressions were additionally sequenced by using a double-stranded DNA cycle sequencing kit (Gibco-BRL). Database searches were performed at the National Center for Biotechnology Information, using the BLAST network service.

Cloning of *M. tuberculosis mtrA* and *mtrB*, visualization of MtrA by using the T7 RNA polymerase/promoter system, and protein purification. The *M. tuberculosis mtrA* and *mtrB* genes were cloned as follows. First, a 1.4-kb *Eco*RI-*Sal*I fragment was identified on genomic blots by hybridization with *P. aeruginosa phoB*, DNA was isolated from the corresponding region on an agarose gel and ligated to pBluescript SK (Stratagene), and positive clones were identified by hybridization. The cloned 1.4-kb *Eco*RI-*Sal*I fragment, which contained only the 5′ two-thirds of *mtrA* and the upstream sequences, was used to isolate a 2.7-kb *Sal*I fragment with the complete *mtrA* gene. This fragment also contained a partial *mtrB* gene. The cloned *mtrA* gene served as a hybridization probe to screen a cosmid *M. tuberculosis* library, and a 7-kb *Eco*RI insert that contained the entire *mtrB* gene and the downstream sequences was subcloned from one of the positive cosmids (T410). For T7 expression and detection of the *mtrA* gene product, the 2.7-kb *Sal*I fragment carrying *mtrA* was digested with *Nco*I and *Sal*I, and the resulting product (with the *Nco*I end converted into a blunt end) was cloned into pT7-5 cut with *Sma*I and *Sal*I. The *Nco*I site in this clone (pT7-5*mtrA*) is located 11 bp upstream of the *mtrA* start codon and includes the putative ribosomal binding site of this gene (Fig. 1). To generate a MalE-MtrA protein fusion, *mtrA* was modified by PCR by using primers RC-12 (5′CATGG ACACC*CAG***CTG**CAAAGGATTTT3′; underlined residues, *Pvu*II site; italicized residues, altered start codon from ATG into CAG; boldface residues, nucleotide alterations relative to the *mtrA* sequence) and RC-13 (5′*TCA*CGGAG GTCCGGCC3′; italicized residues, stop codon of *mtrA*, shown on the antisense strand). The PCR product was cloned into pCRII (Invitrogen), the nucleotide sequence was determined, and *mtrA* was transferred into pMAL-c2 as a *Pvu*II-*Eco*RI fragment (New England Biolabs) to generate the expression clone p*malE-mtr*A. MtrA was purified by two alternative approaches. (i) MtrA was overproduced in the T7 expression system and purified via two chromatographic steps, using procedures for AlgR purification (10). Briefly, sonic lysates were fractionated on MonoQ HR 5/5, using a Pharmacia-LKB fast protein liquid chromatography system, and proteins were eluted with a linear gradient of 0.01 to 0.6 M NaCl in 20 mM Tris-HCl (pH 7.5). Fractions containing MtrA were loaded onto heparin-Sepharose CL-6B bed and eluted with a linear gradient from 0 to 1 M NaCl. The 1 M NaCl fraction was used for further studies. (ii) Overproduction and purification of MalE-MtrA fusion protein were performed by using a protein fusion and purification system (New England Biolabs) as instructed by the manufacturer. The hybrid protein was digested with factor Xa, and liberated MtrA was used for further studies. The polypeptide encoded by the cloned *mtrA* gene was visualized by expression in *Escherichia coli*, using the temperature-inducible T7 expression system (38) and pT7-5*mtrA* and protein labeling with [^{35}S]methionine and [^{35}S]cysteine (1,000 Ci/mmol; DuPont NEN).

Phosphorylation of MtrA. Phosphotransfer between CheA and MtrA was carried out as previously described (10). After incubation of CheA with [γ-^{32}P]ATP, (6,000 Ci mmol^{-1}) for 20 min at room temperature, purified MtrA or AlgR was added. Phosphorylation of MtrA was carried out for 1 h at 37°C, and samples were treated as previously described (10). Products were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and subjected to autoradiography.

Growth and preparation of mycobacterial suspensions for macrophage infection. *M. bovis* BCG and its plasmid-containing derivatives were grown on fresh 7H10 agar for no more than 3 weeks. These bacteria were inoculated into 40 ml of 7H9 liquid medium and grown in 250-ml tissue culture roller bottles (Falcon) at 37°C in 5% CO_2 in humidified air without shaking for 5 to 7 days. Grown in this way, bacteria form a fine precipitate on the bottom of the bottles that can be treated to form a uniform suspension (25). The bacteria were pelleted, resuspended in DMEM with FBS, homogenized by 30 strokes in a Dounce glass homogenizer with a tight pestle, and sonicated in an ultrasonic bath (model 7; Astreson) for 2 min, and large clumps were removed by centrifugation at 200 × *g* for 10 min. A sample from the resulting bacterial suspensions was stained with the *bac*Light stain (Molecular Probes), and bacteria were counted in a Neubauer hemocytometer chamber under a fluorescence microscope to assess uniformity of the single-cell suspensions and to ensure that equal numbers of bacteria were used for macrophage infection in all experiments. Viability of bacterial suspensions was determined by plating serial dilutions of the infecting inoculum on 7H10 agar and was on average 60%, a considerable improvement over 5% in another study (12).

Infection of macrophage monolayers and bacterial recovery from macrophages. For each experiment, J774 macrophage monolayers were established by plating 7.5×10^6 cells per 100-mm-diameter tissue culture petri dish containing glass coverslips when required. After overnight attachment at 37°C in humidified air containing 5% CO_2, macrophages were infected at a multiplicity of infection of 10 to 20 *M. bovis* BCG bacteria per macrophage in the presence of DMEM with 5% FBS. One hour after infection, the inoculating medium was removed, the monolayers were washed three times with prewarmed phosphate-buffered saline (PBS; pH 7.2) to remove noningested bacteria, and fresh medium was added. The medium was replaced every 3 to 4 days of culture. The infected macrophages were incubated from 10 min to 11 days before harvest without any apparent damage to the macrophage monolayer caused by the bacterial infection. At harvest, the monolayers were washed three times with PBS, and the macrophage-containing coverslips, if present, were mounted on slides with PermaFluor (Lipshaw Immunon) for microscopic examination. The remaining macrophages were harvested by scraping from the dishes and centrifugation at 400 × *g* for 5 min at 4°C. Macrophages were broken by homogenization in 20 mM [*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES; pH 7.2)–250 mM sucrose in a Dounce homogenizer until 80% of the macrophages were broken, as determined by visual inspection under a microscope. Serial dilutions from the homogenate were plated on 7H10 to monitor survival of *M. bovis* BCG in macrophages by determining CFU. For flow cytometry, the homogenate was centrifuged (200 × *g*, 10 min, 4°C) to pellet nuclei and unbroken cells, the supernatant was saved, and the bacteria were pelleted by centrifugation at 2,500 × *g* for 15 min at 4°C. The pellet was resuspended in PBS containing 0.1% SDS and 0.1% Triton X-100 to solubilize the remaining macrophage debris and centrifuged (2,500 × *g*, 15 min, 4°C) through a 12% (wt/wt) sucrose cushion. The final bacterial pellet was suspended in PBS for flow cytometry.

Fluorescence microscopy. Infected macrophage monolayers seeded on glass coverslips were examined by epifluorescence microscopy, using an Olympus model BX50 microscope equipped with U-MNIB modular filter cube with a BP470-490 band pass excitation filter, DM 500 dichromatic beam splitter, and BA515-IF barrier emission filter for observation of GFP fluorescence. Images of macrophages containing mycobacteria were recorded on Kodak Ectachrome 400 film with an SC 35 camera through an oil immersion objective (Uplan Fluorite; Olympus). Confocal microscopy was carried out as previously described (12).

Flow cytometry and fluorescence-activated cell sorting. Flow cytometry analysis and fluorescence-activated cell sorting (21) were carried out as previously described (12), using a FACStar Plus system (Becton Dickinson Immunocytometry Systems). Illumination was with a 200-mW 488-nm argon ion laser, and emission light was detected through a 530/30-nm band pass filter. Data were collected on 20,000 individual particles per sample with gating for size to eliminate interference due to potential clumping as published previously (12). For analysis of in vitro-grown *M. bovis* BCG, bacterial cultures were processed as described for macrophage infection.

Nucleotide sequence accession numbers. The *mtrA* and *mtrB* sequences reported here are assigned GenBank accession numbers U01971 and U14909, respectively.

RESULTS

Isolation and characterization of the *mtrA mtrB* locus from *M. tuberculosis* H37Rv. In an attempt to isolate genes encoding signal transduction elements from *M. tuberculosis*, we used a number of previously characterized genes encoding two-component systems from different organisms, including those from bacteria with high GC DNA content similar to the GC content of *M. tuberculosis* (65% [6]) as hybridization probes. One of the probes used, *phoB* from *P. aeruginosa* (1), showed three strong hybridization signals in Southern blot analyses (data not shown). The chromosomal region corresponding to the band displaying the strongest hybridization with *phoB* was cloned via several steps as described in Materials and Methods, the origin of the clone was verified by hybridization with *M. tuberculosis* H37Rv chromosomal DNA, and the insert was subjected to sequence and expression analyses. Global homology searches allowed identification of two genes (Fig. 1 to 3) in the cloned region.

The first gene, *mtrA* (for *M. tuberculosis* response regulator A), showed similarity with typical response regulators, including 209 entries in GenBank, with the *P* value of $<2 \times 10^{-4}$. An alignment of MtrA with a number of homologs (selected on the basis of highest similarity scores or previous characterization at the protein or genetic level) is shown in Fig. 2 to illustrate these relationships. The region showing homology also overlapped with a 435-bp *Hin*cII fragment that provided the strongest hybridization with the *P. aeruginosa phoB* probe used to isolate *mtrA*. The similarities of MtrA with response regulators encompassed the highly conserved N-terminal domain (i.e., corresponding to Asp-13, Asp-53, and Lys-109 of the active site of CheY [37]) and extended to a characteristic conserved C-terminal domain shared with a subset of response regulators termed RO_{II} (30). The best similarity score was

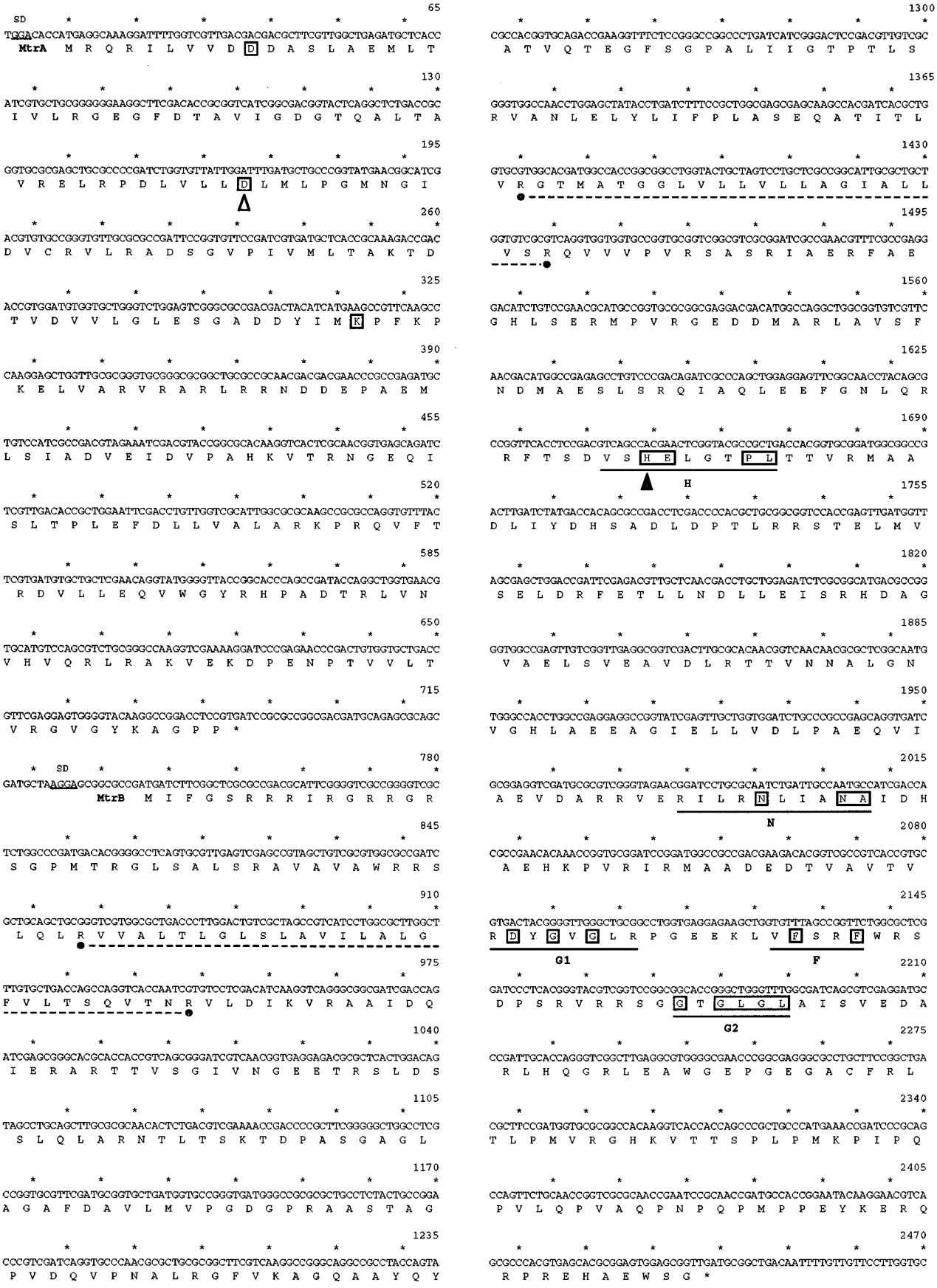

FIG. 1. DNA sequence of the *mtrA* and *mtrB* genes from *M. tuberculosis* H37Rv. SD, putative ribosomal binding site. Boxed residues in MtrA represent amino acids corresponding to the active site Asp and Lys residues in CheY (37). MtrB regions showing similarity with the previously recognized regions of conservation in histidine protein kinases (30, 37) are underlined, and critical residues are boxed. Two putative membrane-spanning regions of MtrB are denoted by dashed lines; dots indicate charged residues demarcating the hydrophobic regions; triangles indicate predicted sites of phosphorylation (open triangle, phosphoaspartate; filled triangle, phosphohistidine).

obtained with AsfQ1 (18), a putative response regulator from *Streptomyces coelicolor* A3(2). This gene stimulates A-factor synthesis in *Streptomyces* spp., in which, depending upon the species, it functions as a microbial hormone controlling aerial mycelium formation or is regarded as a secondary metabolite (17, 18). Additionally, PhoB equivalents from various organisms showed homologies with MtrA, in keeping with the original strategy used to clone *mtrA*.

Interestingly, the predicted product of a sequence termed *phoP*, within the cosmid clone B2168 from *Mycobacterium leprae*, which was entered into GenBank after the deposition of the *mtrA* sequence, also showed similarity with MtrA. However, this sequence, as it appears in GenBank, seemed incomplete because it lacked the N-terminal 30 amino acids which almost invariably contain a highly conserved residue corresponding to Asp-13 of CheY, which forms a part of the acidic pocket within the active site of response regulators. By inserting a nucleotide between positions 36589 and 36588 of the deposited cosmid sequence, the region of homology of *M. leprae* PhoP could be extended to include a common N-terminal domain (Fig. 2), suggesting the presence of a typical response regulator in this region of the *M. leprae* chromosome.

Similar analyses were carried out for MtrB (Fig. 3). MtrB showed similarities to typical histidine protein kinases represented by 123 GenBank entries, with $P < 2 \times 10^{-4}$. The predicted primary structure of MtrB displayed the conserved features of many other histidine protein kinases described by Stock et al. (37) and Parkinson and Kofoid (30) (Fig. 1 and 3). The similarities encompassed the previously recognized conserved motifs including the critical blocks referred to by some investigators as H (containing the histidine residue that serves as the site of autophosphorylation), N, G1, and G2 (30), with regions G1 and G2 resembling glycine-rich nucleotide binding folds. The N-terminal domain of MtrB also appeared to have two putative transmembrane domains containing long uninterrupted stretches of hydrophobic residues demarcated by charged residues (Fig. 1).

Detection of the gene product of *mtrA* and its purification. To confirm that the predicted open reading frame for *mtrA* indeed encodes a polypeptide, the *mtrA* gene was subcloned behind the T7 promoter and its gene product was visualized by labeling with [^{35}S]methionine and [^{35}S]cysteine in the *E. coli* T7 expression system (38). A polypeptide of 30 kDa was detected in these experiments (Fig. 4A). The same system was used to overproduce and purify MtrA by conventional chromatography as described in Materials and Methods. In addition, a fusion protein between MalE and MtrA was generated, and the resulting hybrid protein was overproduced. MtrA was purified following the separation of MtrA from MalE by digestion with factor Xa. The protein product was subjected to N-terminal protein sequence analysis, and the results of these studies (data not shown) confirmed the predicted open reading frame assignment for *mtrA*.

Phosphotransfer between a typical histidine protein kinase and MtrA in vitro. Since one of the goals of this study was to test whether *M. tuberculosis* contains genes participating in phosphotransfer signal transduction processes, the capacity of purified MtrA to undergo phosphorylation was tested. For this

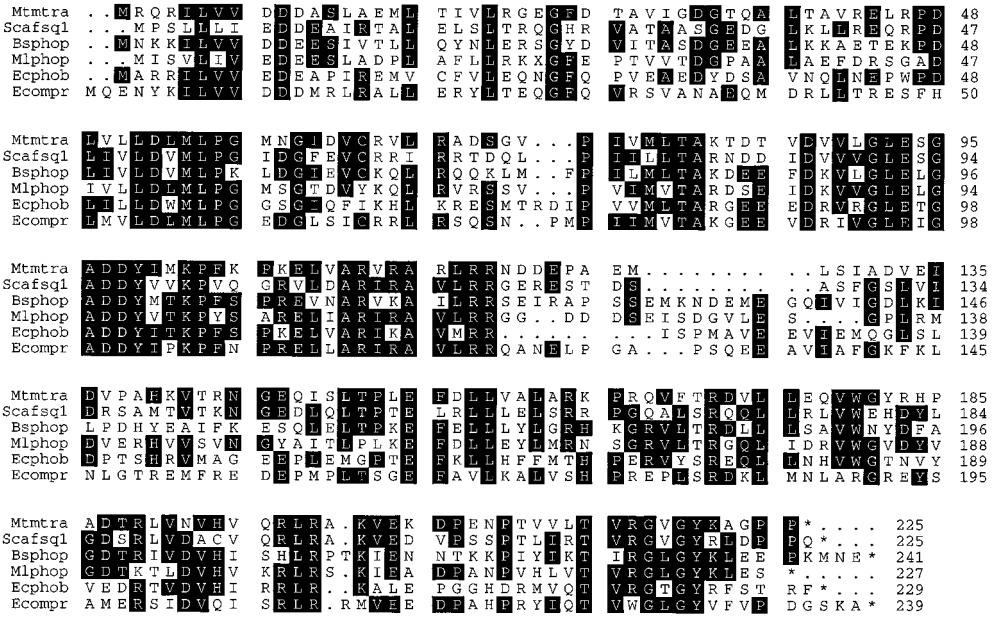

FIG. 2. Alignment of *M. tuberculosis* MtrA (Mtmtra) with a selected number of response regulators: from top to bottom, AfsQ1 from *S. coelicolor* (18), PhoP from *Bacillus subtilis* (33), and PhoB (22) and OmpR from *E. coli* (40). Also included is a translation of the putative *phoP* gene from *M. leprae* after insertion of a nucleotide between positions 36589 and 36588 of the reported cosmid sequence (GenBank accession number U00018), resulting in extension of the region of homology to include a typical N-terminal response regulator domain.

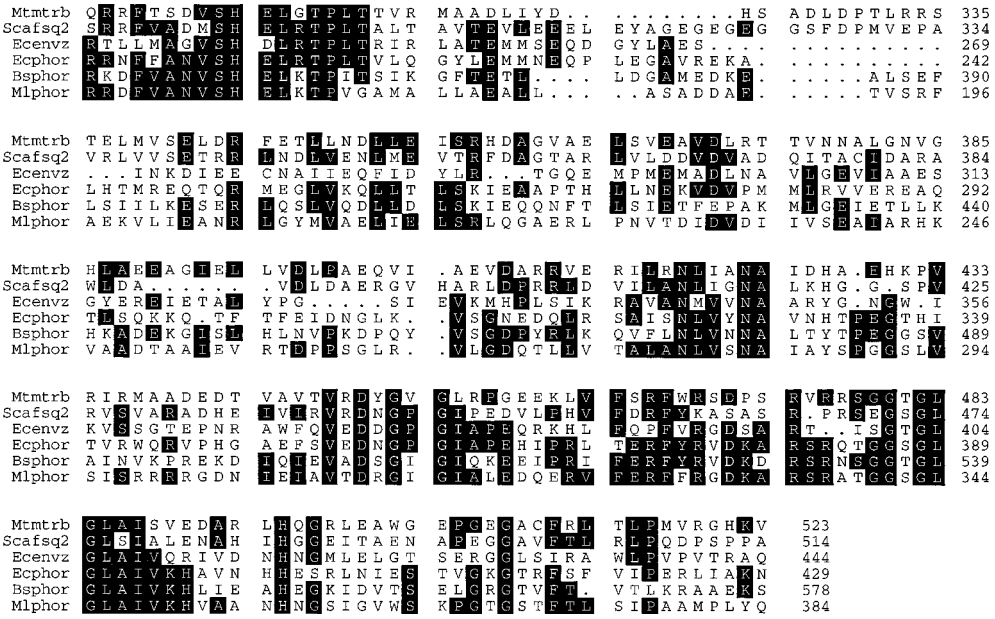

FIG. 3. Alignment of *M. tuberculosis* MtrB (Mtmtrb) with the histidine protein kinases (highly conserved portions) cognate to the response regulators in Fig. 2: from top to bottom, AfsQ2 from *S. coelicolor* (18), EnvZ (7) and PhoR (23) from *E. coli*, PhoR from *Bacillus subtilis* (34), and putative PhoR from *M. leprae* (GenBank accession number U00018).

purpose, a typical histidine protein kinase, CheA, was used. CheA was autophosphorylated and then mixed with MtrA. Although CheA is not a cognate kinase for MtrA, it has been shown to serve efficiently in heterologous phosphotransfer processes, as illustrated by the reaction with another response regulator, AlgR, used as a control in our studies (Fig. 4B) (10). The results with MtrA in this assay (Fig. 4B) demonstrated that the phosphoryl group could be transferred from CheA to MtrA. These findings support the notion that the similarities observed between MtrA and typical response regulators reflect functional homologies and suggest participation of MtrA in putative phosphorylation processes in *M. tuberculosis.*

Studies of *mtrA* expression in macrophages. The infectious cycle of *M. tuberculosis* includes, among other stages, entry and multiplication in host monocytes. Since this process is usually regarded as one of the critical aspects of the *M. tuberculosis* life cycle (9), it seemed pertinent to investigate whether mycobacterial signal transduction systems could be affected by the intracellular environment of the monocyte. While, despite repeated attempts (see Discussion), the targets regulated by MtrA could not be defined, it appeared of interest to test whether expression of *mtrA* was affected by transition from extracellular to intracellular growth in macrophages. To test this possibility, we first examined whether *M. bovis* BCG has the equivalent of *M. tuberculosis mtrA*. The corresponding gene was isolated and shown to have the same sequence in this organism, suggesting that *M. bovis* BCG was suitable as a host to study *mtrA* expression. The promoter region of *M. tuberculosis mtrA* has been previously mapped (8) by transcriptional fusion studies using *xylE* as a reporter gene. *M. bovis* BCG cells harboring the plasmid p*mtrA-gfp*, carrying an *M. tuberculosis mtrA-gfp* transcriptional fusion, and a control plasmid, p*hsp60-gfp*, carrying an h*sp60-gfp* fusion (12), were used to infect J774 macrophage monolayers. GFP fluorescence of the bacilli was monitored by epifluorescence microscopy and quantitated by flow cytometric analyses as described in Materials and Methods. Viability of the input bacterial suspensions ranged from 34 to 70%. At a multiplicity of infection of 10 CFU per macrophage, an average of 9% of the infecting inoculum was ingested by the macrophages. After removal of extracellular bacteria, the infected monolayers were incubated for various times ranging from 10 min to 11 days postinfection. As described previously (12), confocal optical sections confirmed the intracellular location of these bacteria in our experiments (data not shown).

Epifluorescence microscopy images of macrophages with ingested *M. bovis* BCG harboring p*mtrA-gfp* or p*hsp60-gfp* at various times postinfection are shown in Fig. 5. *M. bovis* BGC harboring the *mtrA-gfp* construct increased in fluorescence intensity during incubation inside the macrophage (triplicate samples for each time point were examined in three indepen-

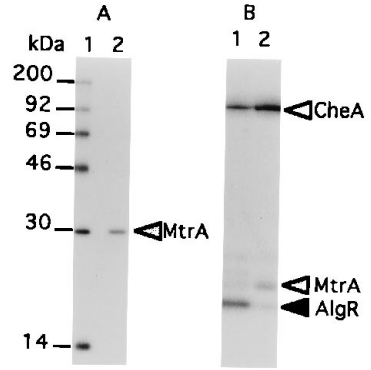

FIG. 4. T7 expression analysis of *mtrA* (A) and in vitro phosphorylation of MtrA (B). (A) [^{35}S]methionine- and [^{35}S]cysteine-labeled polypeptide encoded by the *mtrA* gene was separated by SDS-PAGE and visualized by autoradiography. Lanes: 1, molecular mass standards; 2, 30-kDa polypeptide encoded by the *mtrA* gene. (B) MtrA was incubated with autophosphorylated CheA, and the transfer of phosphate was assessed by electrophoretic separation and autoradiography. Lanes: 1, CheA (open triangle) and AlgR (filled triangle); 2, CheA and MtrA (stippled triangle). Phosphotransfer to AlgR has been previously reported (10) and was included as a positive control.

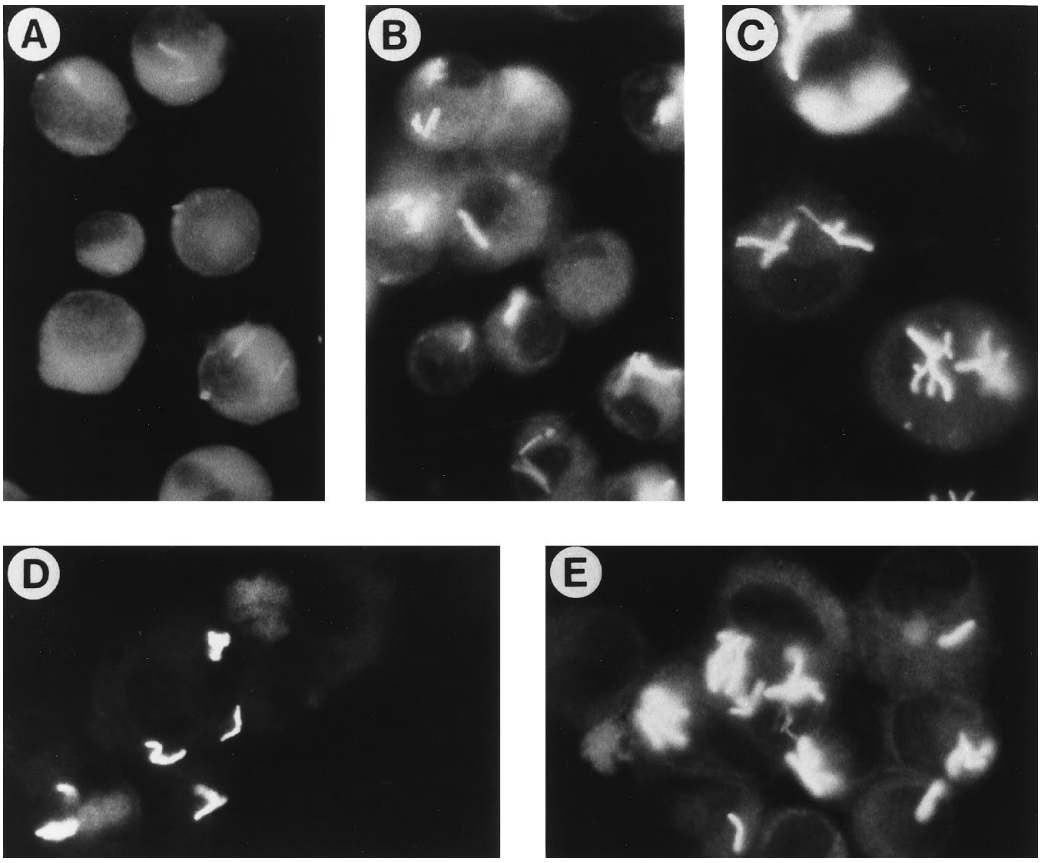

FIG. 5. Monitoring of in vivo expression of *mtrA-gfp* and *hsp60-gfp* in infected macrophages by epifluorescence microscopy. (A to C) Epifluorescence microscopy images showing fluorescence of *M. bovis* BCG harboring the *mtrA-gfp* transcriptional fusion p*mtrA-gfp* in J774 macrophages at 10 min (A), 3 days (B), and 7 days (C) postinfection. (D and E) J774 macrophages infected with *M. bovis* BCG harboring the *hsp60-gfp* transcriptional fusion p*hsp60-gfp* at 10 min (D) and 7 days post-infection (E). Numerous GFP-labeled bacteria are present within the macrophages after 7 days. Note that the fluorescence level of individual bacilli increases in the case of the *mtrA-gfp* fusion.

dent experiments). A visible difference in fluorescence intensity was detectable by day 3 following the infection and increased additionally by day 7 postinfection (Fig. 5). While the intensity of GFP fluorescence in the case of *mtrA-gfp* gradually increased over time, the fluorescence of the *hsp60-gfp* expressing bacteria examined in a parallel experiment did not appear to change significantly. The number of bacilli inside the infected macrophages also appeared to increase over time in all samples (Fig. 5), but analysis by plating at the time of infection and at harvest indicated only a modest increase in *M. bovis* BCG CFU after 7 days (zero to three cell divisions in different samples).

To quantitate the changes in fluorescence of *M. bovis* BCG expressing *mtrA-gfp* after ingestion by the macrophage, samples were subjected to flow cytometric analysis. Macrophages infected with bacteria harboring each of the constructs were processed for flow cytometric analysis immediately after infection and at 7 and 11 days postinfection. Representative distributions of fluorescence intensities from one of three independent experiments are illustrated in Fig. 6. After incubation in the macrophage, the fluorescence of *M. bovis* BGC harboring the *mtrA-gfp* construct increased markedly compared with the initial inoculum (grown in 7H9 medium with ADC) (Fig. 6). Upon 7 days of incubation, the *mtrA-gfp* sample showed a 1-log-unit shift in the maximum of the peak corresponding to fluorescence of the bacteria liberated from the macrophage. In contrast, no significant change (14 to 17% increase) in GFP fluorescence was seen in *M. bovis* BCG containing *hsp60-gfp* over the entire course of the experiment (Fig. 6).

In parallel with the macrophage infection experiments, *M. bovis* BCG bacilli harboring the *mtrA-gfp* or *hsp60-gfp* construct were incubated for 7 days in the medium and under conditions used for macrophage growth to determine if the cell culture medium alone could be responsible for the observed change in fluorescence intensity. The fluorescence of *M. bovis* BGC harboring the *mtrA-gfp* fusion was low and relatively stable, showing a distribution of fluorescence similar to that shown in Fig. 6A in various media tested (DMEM supplemented with 5% FBS; 7H9 or 7H10 supplemented with 10% ADC and 0.05% Tween 80; 7H9 supplemented with 5% FBS). Since growth in these media caused no change in fluorescence of *mtrA-gfp* bacilli, it appears that the induction of *mtrA* expression in experiments carried out in macrophages can be attributed to the intracellular growth.

DISCUSSION

In this work, we present the isolation and initial characterization of a two-component signal transduction system from *M. tuberculosis*. The *mtrA-mtrB* system is to our knowledge the first fully sequenced and biochemically studied example of such regulators in this organism. The *mtrA* gene product has been detected in the *E. coli* T7 expression system. The ability of MtrA to undergo phosphorylation has also been established by

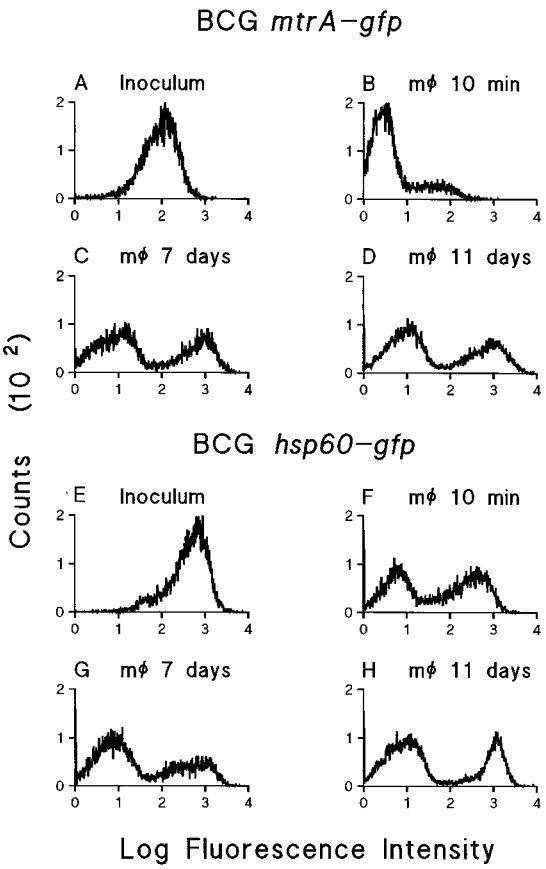

FIG. 6. Induction of *mtrA* expression in *M. bovis*-infected macrophages determined by flow cytometry of GFP fluorescence. Flow cytometric measurements of *M. bovis* BCG harboring the *mtrA-gfp* or *hsp60-gfp* transcriptional fusion were performed at different times postinfection. J774 macrophages were infected with bacilli and incubated for specified periods, after which bacteria were released from macrophages for flow cytometric analysis. (A) *M. bovis* BCG harboring p*mtrA-gfp* used as the inoculum. (B to D) *M. bovis* BCG harboring the *mtrA-gfp* transcriptional fusion liberated from J774 macrophages 10 min (B), 7 days (C), and 11 days (D) postinfection. (E) *M. bovis* BCG harboring p*hsp60-gfp* used as the inoculum. (F to H) *M. bovis* BCG harboring the *hsp60-gfp* transcriptional fusion isolated from J774 macrophages 10 min (F), 7 days (G), and 11 days (H) postinfection. In each experiment with macrophage-grown bacilli (panels B to D and F to H), the peak on the right corresponds to morphologically distinguishable *M. bovis* bacilli as described previously (12). This was verified by fluorescence-activated cell sorting and visual inspection of the sorted material by epifluorescence microscopy. The total number of input particles in each sample was standardized to 20,000, but the distribution between the two peaks varied from sample to sample. Fluorescence intensity is judged only by the *y* coordinate of the maximum of the peak corresponding to the bacilli (12). Note the shift toward higher intensity levels in *M. bovis* BCG harboring *mtrA-gfp* upon incubation in macrophages.

using CheA as a phosphodonor. While the putative MtrA-cognate kinase MtrB has not been subjected to a similar biochemical analysis, the close genetic linkage of *mtrA* and *mtrB* suggests that their gene products may represent parts of the same two-component system. Phosphorylations are frequently utilized for transient and sometimes rapid environmental responses, and the presence in *M. tuberculosis* of factors that can undergo such modifications, as shown in the case of MtrA, suggests that this organism has the capacity for such processes.

Given what has been learned about the two-component regulatory systems in general (16, 24, 26, 30, 40) and the available information on the subset of response regulators (RO_{II}) to which MtrA belongs (30), MtrA may act as a transcriptional activator. Repeated attempts to disrupt *mtrA* on the chromosome of *M. tuberculosis* or *M. bovis* BCG have been unsuccessful to date. While it is generally acknowledged that homologous recombination is difficult in slowly growing mycobacterial species (3, 28), this may be further compounded by the possibility that a chromosomal disruption of the *mtrA-mtrB* locus could adversely affect viability of any recombinant bacilli. While similarity in signal transduction systems does not necessarily indicate functional equivalence, it may be of interest that MtrA and MtrB display significant similarity to PhoB and PhoR from a number of different species. However, despite repeated intergeneric complementation experiments, we were not able to demonstrate a functional equivalence between *mtrA* and *phoB* by using several *phoB* mutants of *E. coli* or *P. aeruginosa*. There was also no conclusive evidence whether the *mtrA-mtrB* system affected the production of the putative phosphate transporter PhoS (the 38-kDa antigen). In related attempts to investigate whether the reduced levels of *phoS* expression in *M. bovis* BCG relative to *M. tuberculosis* (39) could be due to a regulatory defect, the level of PhoS in *M. bovis* BCG harboring the cosmid T410, which carries the complete *M. tuberculosis mtrA-mtrB* region, was analyzed. Using antibody TB71 against the 38-kDa antigen, we could not detect a change in the amounts of PhoS in *M. bovis* BCG with or without plasmid-borne *mtrA-mtrB* (data not shown). Furthermore, sequence analysis indicated that *M. bovis* BCG had an intact *mtrA* gene (unpublished data).

Considering that MtrA-MtrB showed greatest similarity to AfsQ1-AfsQ2 and the link of the latter to developmental phenomena in *Streptomyces* spp., we were prompted to examine the possibility that *mtrA-mtrB* is involved in life cycle changes experienced by *M. tuberculosis*. In a simplified scheme, the growing *M. tuberculosis* can be viewed as alternating between intracellular multiplication in host cells, extracellular survival and replication in liquefied caseous foci, and survival during aerosolization and transmission in droplet nuclei (9). On the basis of these considerations and the fact that many two-component systems are subject to autoregulatory and other control processes (16, 26, 27, 37), including effects on gene expression during intercellular growth in macrophages (16, 27), we explored whether expression of the *mtrA-mtrB* system itself might respond to entry into the macrophage and intracellular environment. In our previous studies (8), activity of the *mtrA* promoter was examined by using *xylE* as a reporter gene in *M. smegmatis* as a host, and limited responses to growth conditions were observed. The recent development of GFP fluorescence-based in vivo expression technology (12, 20) permitted us to take a direct approach in addressing the question of expression in macrophages. In experiments reported here, a significant increase in fluorescence intensity of the bacilli carrying *mtrA-gfp* was observed in macrophages.

Several two-component systems have been implicated in the control of virulence of intracellular pathogens, as in the case of the PhoP-PhoQ system in *Salmonella typhimurium* (16, 27), opening interesting possibilities for MtrA-MtrB in *M. tuberculosis*. Genes that are selectively expressed or regulated during growth in the macrophage are likely to be involved in intracellular survival and thus possibly enhance virulence of *M. tuberculosis*. Strategies to identify the target gene or genes activated by *mtrA* are currently under development. These studies will help determine whether MtrA and MtrB serve as a recognition system setting in action changes relevant for intracellular survival and growth of *M. tuberculosis.*

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