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An Essential Two-Component Signal Transduction System in *Mycobacterium tuberculosis*

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The bacterial two-component signal transduction systems regulate adaptation processes and are likely to play a role in *Mycobacterium tuberculosis* physiology and pathogenesis. The previous initial characterization of an *M. tuberculosis* response regulator from one of these systems, *mtrA-mtrB*, suggested its transcriptional activation during infection of phagocytic cells. In this work, we further characterized the *mtrA* response regulator from *M. tuberculosis* H37Rv. Inactivation of *mtrA* on the chromosome of *M. tuberculosis* H37Rv was possible only in the presence of plasmid-borne functional *mtrA*, suggesting that this response regulator is essential for *M. tuberculosis* viability. In keeping with these findings, expression of *mtrA* in *M. tuberculosis* H37Rv was detectable during in vitro growth, as determined by S1 nuclease protection and primer extension analyses of mRNA levels and mapping of transcript 5′ ends. The *mtrA* gene was expressed differently in virulent *M. tuberculosis* and the vaccine strain *M. tuberculosis* var. *bovis* BCG during infection of macrophages, as determined by monitoring of *mtrA-gfp* fusion activity. In *M. bovis* BCG, *mtrA* was induced upon entry into macrophages. In *M. tuberculosis* H37Rv, its expression was constitutive and unchanged upon infection of murine or human monocyte-derived macrophages. In conclusion, these results identify *mtrA* as an essential response regulator gene in *M. tuberculosis* which is differentially expressed in virulent and avirulent strains during growth in macrophages.

Tuberculosis remains the leading cause of death in the world from a single infectious agent (2). The capacity of *Mycobacterium tuberculosis* to establish infection within an individual and efficiently disseminate within the human population is mediated in part by its ability to survive within professional phagocytic cells, remain dormant over long periods of latent infection, and resume growth upon disease reactivation (27). The physiological and environmental signals during periods of active disease, dormancy, or disease reactivation are likely to contribute to *M. tuberculosis* adaptation during various stages of infection. One well-recognized class of ubiquitous bacterial regulatory elements associated with signal recognition and adaptive responses is that of the two-component signal transduction systems. Bacterial two-component systems regulate various functions, including transient adaptations, developmental phenomena, and production of secondary metabolites (reviewed in reference 16). In pathogenic organisms, two-component systems can also regulate expression of virulence determinants or factors that contribute to disease pathogenesis (reviewed in references 16 and 30). In addition to the majority of two-component systems, which modulate nonvital albeit important cellular functions, a limited number of essential two-component systems have also been described. Such systems, although rare, have been shown to regulate genes involved in cell cycle control (29) and membrane permeability (23).

M. tuberculosis encodes a number of two-component signal transduction systems. The MtrA-MtrB system was the first such system to be characterized in the tubercle bacillus (5, 7, 35). Since then, an additional 11 complete and 8 unlinked sensor kinase and response regulator homologs have been identified in the *M. tuberculosis* H37Rv genome (4, 12, 14, 21, 22, 33). Some of these two-component systems appear to be differentially regulated during growth within cultured macrophages in vitro. For example, expression of the *mtrA* response regulator (*Rv3246c*), which has been studied in *M. bovis* BCG, is induced in infected murine macrophages (7, 35). In addition, cDNAs corresponding to transcripts encoding the *prrA* response regulator (*Rv0903c*) and the sensor kinase *prrB* (*Rv0902c*) have been recovered from *M. tuberculosis* grown in human peripheral blood monocyte-derived macrophages but not from bacteria grown in standard laboratory medium (12). These limited examples reflect the preliminary nature of the initial analyses of *M. tuberculosis* two-component systems. In continuation of our characterization of the *mtrA-mtrB* system, we attempted to disrupt the *mtrA* gene in *M. tuberculosis*. Here we present data suggesting that *mtrA* is an essential gene in *M. tuberculosis* H37Rv. We also report the mapping of the 5′ end of the *mtrA* mRNA and its in vivo expression profiles in *M. tuberculosis* H37Rv and *M. bovis* BCG.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and electrotransformation. *M. tuberculosis* H37Rv (ATCC 27294) and *M. bovis* BCG Pasteur (ATCC 27291) were used. All transformations done with *Escherichia coli* were performed with strain DH5α. Mycobacteria were grown under standard conditions in Middlebrook 7H9 broth or on Middlebrook 7H10 agar (Difco Laboratories) supplemented with 0.5% glycerol, 10% ADC or OADC (oleic acid-albumin-dextrose-catalase) (Difco) and 0.05% Tween 80 (Sigma) at 37°C in the presence of 5% CO_2. *E. coli* was grown in LB medium (Difco) and incubated at 37°C. When required, Middlebrook or LB medium was supplemented with 25 or 50 μg of kanamycin sulfate (Sigma) per ml, 50 or 200 μg of hygromycin B (Boehringer Mannheim) per ml, 25 or 100 μg of streptomycin sulfate (Sigma) per ml, and 2 or 10% sucrose, respectively. Preparation of electrocompetent cells and transformation of *M. tuberculosis* were performed as previously described (18).

Construction of plasmid vectors. Plasmid p*mtrA-gfp* has been described previously (7). Plasmid pTZ113 was used for the disruption of *mtrA* and was constructed as follows. A 2.7-kb *Sal*I fragment containing the entire *mtrA* gene and the 5′ end of *mtrB* was filled in by treatment with Klenow enzyme and ligated into the *Sma*I site of pSM243, a mycobacterial suicide vector carrying the *sacB* gene. Next, a 1.2-kb *Nhe*I-*Spe*I fragment carrying the Km^r gene from pMV206 (31) was filled in and cloned into a blunt-ended *Bgl*II site in *mtrA*. Finally, a 2.0-kb *Nhe*I-*Xba*I fragment encoding *xylE* from pHSX-1 (5) was ligated into the *Xba*I site of the pSM243-derived polylinker. Temperature-sensitive (ts), *mtrA*$^+$-com-

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plementing plasmid pTZ178 was constructed by first ligating the *Sal*I fragment carrying *mtrA* into the *Sal*I site of pUC12 to create pTZ100. An *Spe*I-*Not*I fragment encoding Hyg^r from pOLYG, a derivative of p16R1 (11), was filled in and ligated into the *Sma*I site of pTZ100 to create pTZ175. Finally, a filled-in *Eco*RV-*Kpn*I fragment encoding the ts origin of replication from pCG63 (13) was ligated into the *Sca*I site of pTZ175 to create pTZ178. pTZ195 is a derivative of pTZ178 that lacks the *Sal*I fragment encoding *mtrA*. Plasmid pTZ199 was constructed by ligating a 3.4-kb *Spe*I fragment encoding Str^r from pSM240 into an *Spe*I-*Nhe*I fragment carrying *xylE*$^+$ from pHSX-1 (5).

Genetic scheme for *mtrA*::Km^r gene replacement. Plasmid pTZ113 (*mtrA*::Km^r *sacB*$^+$ *xylE*$^+$) was used for allelic exchange of *mtrA*$^+$ with *mtrA*::Km^r in *M. tuberculosis* H37Rv via a two-step recombination process (Fig. 1). In the first step (integration), pTZ113 was transformed into *M. tuberculosis* H37Rv and recombinants were selected on 7H10 agar containing kanamycin. Merodiploid transformants were distinguished from spontaneous Km^r mutants by spraying colonies with 100 mM catechol (Fisher Scientific) (5). Transformants expressing *xylE* (detected as yellow colonies upon spraying with catechol) were screened for legitimate single-crossover homologous recombination by PCR using primers *mtrA*upstm2 (5′-CTGACCAAGCTGACCAAGGA-3′), which is a primer upstream of *mtrA* (and not carried on pTZ113), and KmUP2 (5′-GTAAGCAGA CAGTTTTATTGTTCATGA-3′), which is a primer specific to and amplifying out of the Km^r cassette. *mtrA*::Km^r-*mtrA*$^+$ merodiploids resulting from single-crossover homologous recombination were subsequently resolved of pTZ113 to leave *mtrA*::Km^r or *mtrA*$^+$ in the chromosome by growth on 7H10 agar (with or without kanamycin) and sucrose, respectively (28). Colonies resistant to sucrose and white upon spraying with catechol (loss of *sacB* and *xylE* markers) were further screened by PCR using primers that flank the Km^r disruption site in *mtrA* (RC4 [5′-ACGTACCGGCGCGCACAAGGT-3′] and RC13 [5′-TCACGGAG GTCCGGCC-3′]) and primers that amplify an internal portion of *xylE* (*xylE*start [5′-ATGAACAAAGGTGTAATGCG-3′] and *xylE*end [5′-GCGGTCGTGGTA AAAGATCG-3′]).

Addition of *mtrA*$^+$ to *mtrA*::Km^r-*mtrA*$^+$ merodiploid strains. *mtrA*::Km^r-*mtrA*$^+$ merodiploid recombinants resulting from single-crossover homologous recombination of pTZ113 into the *M. tuberculosis* H37Rv chromosome were transformed with pTZ178 [*mtrA*$^+$ *oriM* (ts) Hyg^r], a conditionally replicating plasmid carrying *mtrA*$^+$. Resolution of pTZ113 in these strains to leave *mtrA*::Km^r or *mtrA*$^+$ in the chromosome was achieved by plating on 7H10 agar (with or without kanamycin) containing sucrose and hygromycin and growth at 30°C (permissive temperature for pTZ178 replication). The resulting recombinants were subjected to the screens previously described. Because pTZ178 contained a ts origin of replication, loss of the *mtrA*$^+$ complementing plasmid in *mtrA*::Km^r mutants was attempted by growing strains at the nonpermissive temperature of 39°C. In addition, loss of pTZ178 was also attempted by introduction of a second plasmid, pTZ199 (pMV261 *oriM xylE*$^+$ Str^r), carrying the same origin of replication as pTZ178.

DNA extraction and Southern analysis. Mycobacterial genomic DNA was prepared as previously described (18). A 4-μg sample of genomic DNA was digested overnight with *Eco*RI (Gibco BRL), separated by electrophoresis on a 0.8% agarose gel, transferred onto a Duralon-UV membrane (Stratagene), and used in subsequent high-stringency hybridization and washing steps (26). An *mtrA*-specific probe was generated by random-primed labeling (Gibco) with [α-^{32}P]dCTP (3,000 Ci mmol^{-1}; NEN Dupont) using PCR products generated with oligonucleotides RC4 and RC10 (5′-CCCATCACCCGGCACC-3′).

S1 nuclease protection and primer extension. Total RNA from *M. tuberculosis* H37Rv was isolated as previously described (8). To generate a uniformly labeled single-stranded DNA (ssDNA) probe for S1 nuclease protection, a 1.7-kb *Sal*I-*Pst*I fragment carrying the *M. tuberculosis* H37Rv *mtrA* gene and upstream sequences was directionally cloned into an M13-based phagemid vector (24). ^{32}P-radiolabeled ssDNA probes were prepared (25) using primers *mtrA*S5 (5′-TCG CCGATGACCGCGGTGTC-3′) and *mtrA*S6 (5′-AGCGGCTACTCCGCGGT GTCGAAGCCTTCC-3′). Probes generated from primer *mtrA*S6 contain a 10-bp overhang (underlined nucleotides) at the 5′ end that is not homologous to *mtrA* mRNA. Radiolabeled ssDNA polymerization products were digested with *Age*I, heat denatured in formamide, and gel purified. Hybridization reactions were performed using 75 μg of total RNA from *M. tuberculosis* H37Rv, and S1 nuclease protection was carried out as previously described (25). Products of S1 digestion were analyzed on sequencing gels and compared with the corresponding sequencing ladders to locate mRNA 5′ ends. For primer extension, primer *mtrA*S8 (5′-TCCCCCCGCAGCACGATGGTGAGCATCTCA-3′) was end labeled with [γ-^{32}P]ATP (6,000 Ci mmol^{-1}; NEN Dupont) and purified on a Sephadex G-25 spin column (Boehringer Mannheim). Radiolabeled primer was added to 10 μg of total *M. tuberculosis* H37Rv RNA in hybridization buffer (0.5 M KCl, 0.25 M Tris-HCl, pH 8.3), and aliquots were denatured, annealed, and extended by the addition of 0.1 M dithiothreitol, 2.5 mM deoxynucleoside triphosphates, reverse transcription buffer, and Superscript II reverse transcriptase (Gibco). Extension reactions were carried out at 44°C for 45 min, and samples were loaded on a sequencing gel alongside the corresponding sequence ladder.

Preparation of mycobacteria, infection of macrophage monolayers, and fluorescence microscopy. *M. tuberculosis* H37Rv or *M. bovis* BCG Pasteur was grown in static cultures until cells reached mid-exponential phase (optical density at 600 nm of 0.5). Bacterial cells were prepared for macrophage infection by washing in phosphate-buffered saline (PBS; pH 7.2) and resuspension either in Dulbecco's modified Eagle's medium (Bio Whittaker) supplemented with 10% fetal bovine serum (Hyclone) and 4 mM L-glutamine (Bio Whittaker) or in RPMI 1640 medium (Bio Whittaker) supplemented with 5% human AB serum (Sigma) and 2 mM L-glutamine. Single-cell bacterial suspensions were obtained by vortexing bacteria with 3-mm glass beads (Fisher), low-speed centrifugation, and passage of the resulting supernatant through a 5-μm-pore-size filter (Micron Separations Inc.). The number of organisms was determined by staining with Bac-Light (Molecular Probes) and counting in a hemocytometer. Mycobacteria were used to infect murine BALB/c macrophage cell line J774A (ATCC TIB-67) or human macrophages derived from peripheral blood monocytes (3) obtained from the American Red Cross. J774 cells and human monocyte-derived macrophages were cultured before infection and maintained during infection in supplemented Dulbecco's modified Eagle's medium and RPMI medium, respectively. Both murine and human macrophage monolayers were maintained at 37°C in humidified air containing 5% CO_2. For infections, macrophage monolayers were established by plating 10^5 cells per well in 12-well tissue culture plates (Corning) containing no. 1 thickness, 18-mm-diameter glass coverslips (Fisher). Macrophages were infected with mycobacteria at a multiplicity of infection of 10 bacilli per macrophage. Macrophages were allowed to take up bacteria for 2 h before extracellular bacteria were removed by washing in PBS. Macrophages were incubated for 2 h, 3 days, or 5 days before harvest with no apparent damage. At harvest, macrophage monolayers were washed in PBS, fixed in 3.8% paraformaldehyde, and mounted on glass slides with Permafluor (Lipshaw Immunon). Epifluorescence images were captured using a Kodak Kaf 1400-2 Olympix camera connected to an Olympus BX60 microscope. Images were captured with a shutter speed of 500 ms and analyzed using Espirit software (Life Sciences Resources). NIH Image (version 1.62; National Institutes of Health) was used to quantitate mean pixel density from individual bacilli present within monolayers. At the settings used, macrophage autofluorescence was not observed. Statistical analysis (analysis of variance [ANOVA] and Fisher's protected least significant difference) was performed with ANOVA (version 1.11; Abicus Software).

RESULTS AND DISCUSSION

Gene replacements with *mtrA*::Km^r in *M. tuberculosis* H37Rv. To further examine the role of *mtrA* in *M. tuberculosis*, we set out to disrupt *mtrA* in strain H37Rv. We constructed a mycobacterial suicide plasmid, pTZ113 (see Materials and Methods), that carried a copy of *mtrA* disrupted by a Km^r cassette, the counterselectable marker *sacB*, and the *xylE* gene as a convenient scorable marker for subsequent recombination steps. Following electroporation of pTZ113 into *M. tuberculosis* H37Rv, we obtained recombinants expressing *xylE* (detected as yellow colonies upon spraying with catechol), of which 2.6% had undergone legitimate single-crossover homologous recombination into the chromosome, resulting in a tandem *mtrA*::Km^r-*mtrA*$^+$ merodiploid (Fig. 1A and Table 1, row A). The low frequency of legitimate single-crossover homologous recombination was most likely a result of illegitimate integration of pTZ113 into the *M. tuberculosis* genome (1, 19). Subsequent attempts to produce the desired *mtrA*::Km^r gene replacements by resolving the *mtrA*::Km^r-*mtrA*$^+$ merodiploids via a second crossover after selection against *sacB* failed (Fig. 1B and Table 1, row B). None of the colonies recovered as resistant to sucrose (loss of the *sacB* marker) and not expressing *xylE* (white upon spraying with catechol) were true double-crossover recombinants and most likely harbored other types of mutations eliminating or precluding *sacB* and *xylE* activity (data not shown). The inability to obtain an *mtrA*::Km^r gene replacement was not simply the result of inefficient resolution by homologous recombination, because double-crossover recombinants which had lost the plasmid moiety along with *mtrA*::Km^r, leaving the wild-type copy of *mtrA* in the chromosome, occurred efficiently (Fig. 1C and Table 1, row C).

The *mtrA* gene is an essential response regulator in *M. tuberculosis*. To test further whether the encountered difficulties in inactivating *mtrA* could be explained by its potentially essential function, we introduced into the *mtrA*::Km^r-*mtrA*$^+$ merodiploids plasmid pTZ178 containing an *mtrA*$^+$ copy on a conditionally replicating (ts) mycobacterial shuttle vector and repeated the selection procedure for *mtrA*::Km^r gene replace-

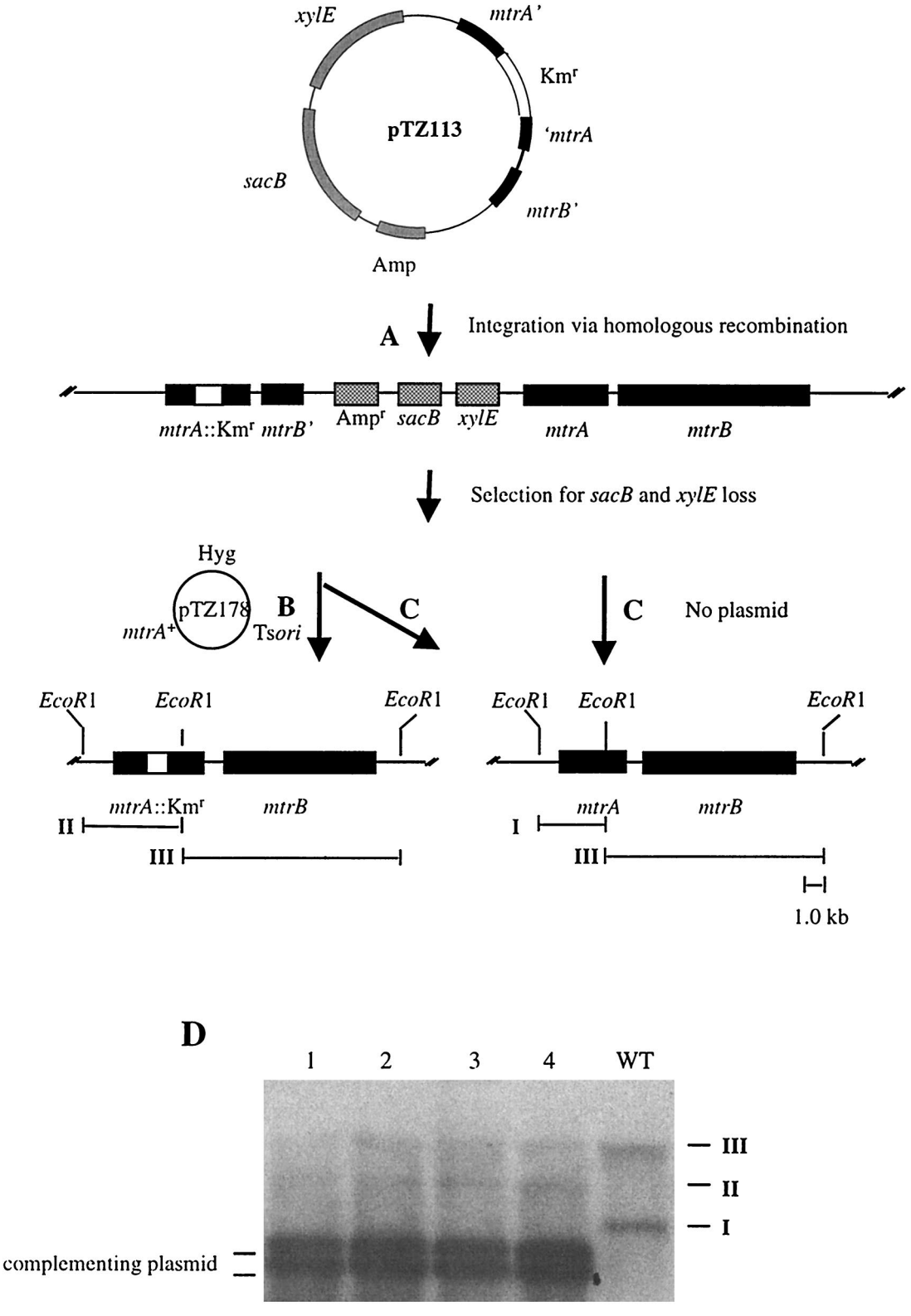

FIG. 1. Genetic scheme for *mtrA* gene replacements in *M. tuberculosis*. (A) Mycobacterial suicide plasmid pTZ113 was integrated into the chromosome of *M. tuberculosis* H37Rv via legitimate single-crossover homologous recombination. (B and C) In the presence of plasmid pTZ178 (*mtrA*$^+$), the merodiploid strain can be resolved upon selection on sucrose to either side of the Km^r cassette to leave the *mtrA*::Km^r disruption in the chromosome (B) or leave wild-type *mtrA*$^+$ in the chromosome (C). In the absence of pTZ178 (no plasmid), only strains retaining wild-type *mtrA*$^+$ are recovered (C). (D) Southern blot analysis of four independent strains with *mtrA*::Km^r allelic replacements on the chromosome obtained in the presence of pTZ178 (scheme B). *Eco*RI fragments: I, 3.4 kb; II, 4.6 kb; III, 7.1 kb. WT, wild type.

TABLE 1. Screening for *mtrA*::Km^r gene replacements in *M. tuberculosis* H37Rv

Row	Step[a]	Parental strain plasmid(s)[b]	Selection/ screen	Expected genotype	Resulting strain plasmid[c]	No. of colonies screened	% Legitimate events[d]
A	Integration	pTZ113 (*mtrA*::Km^r *xylE*$^+$ *sacB*$^+$)	Km^r/XylE$^+$ Suc^s	*mtrA*::Km^r-*mtrA*$^+$	Cointegrate	191	2.6
B	Resolution	Cointegrate	Suc^r/XylE$^-$ Km^r	*mtrA*::Km^r	—	500	0
C	Resolution	Cointegrate	Suc^r/XylE$^-$ Km^s	*mtrA*$^+$	—	600	100
D	Resolution	pTZ178 (*mtrA*$^+$ Hyg^r)	Suc^r/XylE$^-$ Km^r	*mtrA*::Km^r	pTZ178 (*mtrA*$^+$ Hyg^r)	200	96
E	pTZ178 loss	pTZ178 (*mtrA*$^+$ Hyg^r)	39°C/Hyg^s	*mtrA*::Km^r	—	100	0
F	pTZ193 loss	pTZ193 (Hyg^r)	39°C/Hyg^s	*mtrA*::Km^r-*mtrA*$^+$	—	200	100
G	pTZ178 loss	pTZ178 (*mtrA*$^+$ Hyg^r), pTZ199 (Str^r)	Str^r/Hyg^s	*mtrA*::Km^r	pTZ199 (Str^r)	100	0
H	pTZ193 loss	pTZ193 (Hyg^r), pTZ199 (Str^r)	Str^r/Hyg^s	*mtrA*::Km^r-*mtrA*$^+$	pTZ199 (Str^r)	100	100

[a] Steps involved in the construction or resolution of the *mtrA* disruption. Parental strains were *M. tuberculosis* H37Rv (row A), *M. tuberculosis* H37Rv *mtrA*::Km^r-*mtrA*$^+$ *sacB*$^+$ *xylE*$^+$ (rows B and C), *M. tuberculosis* H37Rv *mtrA*::Km^r-*mtrA*$^+$ (pTZ178) (rows D, E, and G), and *M. tuberculosis* H37Rv *mtrA*::Km^r-*mtrA*$^+$ (pTZ193) (rows F and H).

[b] Plasmids were pTZ113 (pUC18 *mtrA*::Km^r *sacB*$^+$ *xylE*$^+$ [Fig. 1]), pTZ178 [pUC12 *oriM* (ts) Hyg^r *mtrA*$^+$], pTZ193 [pUC12 *oriM* (ts) Hyg^r], and pTZ199 (pMV261 *oriM xylE*$^+$ Str^r). Cointegrate refers to pTZ113 integrated on the chromosome of H37Rv via single-crossover homologous recombination with the *mtrA* gene.

[c] —, no plasmid.

[d] A legitimate event is achievement of the expected genotype.

ments. This time, in the presence of plasmid-borne *mtrA*$^+$, true *mtrA*::Km^r gene replacements were obtained via a second crossover on the chromosome (Fig. 1B and Table 1, row D). Southern hybridization analysis performed on four randomly selected recombinants confirmed the replacement of *mtrA*$^+$ with the *mtrA*::Km^r allele on the *M. tuberculosis* chromosome (Fig. 1D). Two *Eco*RI chromosomal fragments in wild-type *M. tuberculosis* H37Rv hybridized with the *mtrA* probe (3.4- and 7.1-kb fragments I and III, respectively). In *mtrA*::Km^r recombinants, fragment I was lost but, instead, a new fragment (fragment II) of 4.6 kb (corresponding to 3.4-kb *Eco*RI fragment I carrying the 1.2-kb Km^r insert) was detected in each of the four recombinants tested. These strains, however, also harbored a plasmid (pTZ178) borne wild-type *mtrA* gene.

Because pTZ178 carried a mycobacterial ts origin of replication, we next tried to eliminate the plasmid by growing the *mtrA*::Km^r (pTZ178) strains at the nonpermissive temperature of 39°C. However, none of the colonies arising following growth at the nonpermissive temperature lost the complementing plasmid (Table 1, row E). In contrast, loss of pTZ193 (a derivative of pTZ178 lacking the *mtrA* gene) from the *mtrA*::Km^r-*mtrA*$^+$ merodiploid parental strain was observed at high frequency (100%) upon growth at 39°C (Table 1, row F). Because two plasmids containing the same origin of replication cannot be maintained simultaneously in the same cell, we also tried to eliminate pTZ178 from *mtrA*::Km^r mutants by introduction of plasmid pTZ199. This approach also failed to cure the *mtrA*$^+$ plasmid from *mtrA*::Km^r mutants despite the fact that the merodiploid parental strain carrying pTZ193 could be cured of this plasmid (Table 1, rows G and H). Based on these experiments, we conclude that *mtrA* encodes a response regulator that is essential for *M. tuberculosis* viability in vitro. The viability-associated function resided within the *mtrA* gene and was not due to polar effects on *mtrB*, as it was possible to knockout *mtrA* on the chromosome in the presence of the *mtrA*$^+$ complementing plasmid, which did not contain a complete *mtrB* gene (Fig. 1D).

Expression of *mtrA* in *M. tuberculosis* grown outside the host. The inability to recover viable *mtrA* mutants would require that *mtrA* be expressed in vitro. Expression of the *mtrA* gene is inducible in *M. bovis* BCG during growth in cultured J774 macrophages (35). However, this does not preclude the possibility that *mtrA* is expressed at baseline levels in vitro outside the host. To test this possibility, we analyzed *mtrA* transcription and mapped *mtrA* mRNA 5′ ends in *M. tuberculosis* H37Rv by S1 nuclease protection and primer extension analyses. Total cellular RNA was isolated from bacteria grown in 7H9 medium and hybridized with a uniformly labeled ssDNA probe, and the products of the hybridization reaction were digested with S1 nuclease. In keeping with our prediction that *mtrA* was expressed during in vitro growth, a band of protection corresponding to *mtrA* transcripts was observed (Fig. 2A). The 5′ end of the protected fragments corresponded to the *mtrA* initiation codon. Similar results were obtained using a probe (Fig. 2D) generated with a primer that contained a 5′ (10-bp) overhang that did not correspond to the *mtrA* sequence. The addition of the heterologous 10-bp overhang sequence resulted in the corresponding reduction in the size of the protected fragment obtained upon S1 nuclease treatment (Fig. 2B), confirming that the assigned transcript was *mtrA* specific and indicating that its 5′ end coincided with the translational initiation site. Due to the intrinsic heterogeneity of the products of uniformly labeled S1 nuclease probes, which introduced some uncertainty regarding the exact position of the mRNA 5′ end, it was important to test whether the *mtrA* mRNA 5′ end included the *mtrA* translational start. With this aim, we performed primer extension analysis. The results of these experiments indicated that the translation and transcriptional initiation start sites of *mtrA* overlap (Fig. 2C). Thus, in *M. tuberculosis* H37Rv, *mtrA* is expressed in vitro from a transcript with the 5′ mRNA end overlapping the translational start site. Overlapping transcriptional and translational start sites have been identified for several other mycobacterial genes, including the major *oxyR* transcript of *M. leprae* (6) and the *furA* promoter from *M. tuberculosis* (unpublished data). In addition, a large number of genes from actinomycetes, a phylogenetic group closely related to mycobacteria, also contain overlapping transcriptional and translational sites (32). In conclusion, the in vitro expression of *mtrA* in *M. tuberculosis* H37Rv was in keeping with our finding that *mtrA* is essential for the viability of this organism.

The *mtrA* promoter is induced in *M. bovis* BCG but is constitutively active in *M. tuberculosis* H37Rv within macrophages. To examine *mtrA* expression in *M. tuberculosis* during intracellular growth, we tested whether the *mtrA* induction previously detected in *M. bovis* BCG could also be observed in *M. tuberculosis* H37Rv during infection of J774 murine macrophages. As reported previously, green fluorescent protein fluorescence in *M. bovis* BCG carrying p*mtrA-gfp* became detectable after 3 days of incubation in J774 cells (Fig. 3A to C) (35). However, green fluorescent protein fluorescence from p*mtrA-gfp* was bright in *M. tuberculosis* H37Rv even prior to infection and no further induction was observed during growth in J774 murine

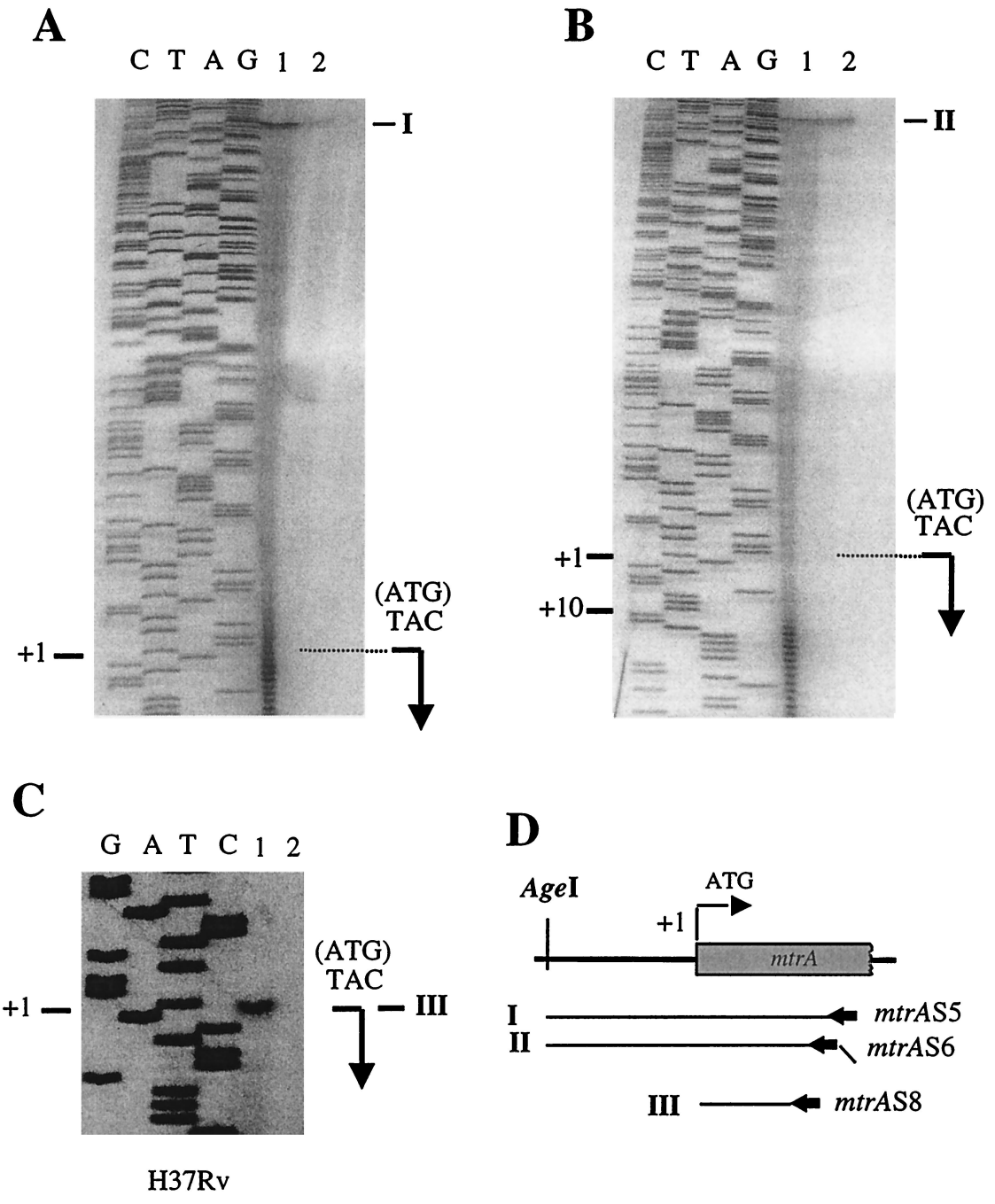

FIG. 2. S1 nuclease mapping and primer extension analysis of the *mtrA* promoter. (A and B) S1 nuclease protection was performed with total cellular RNA from *M. tuberculosis* H37Rv and uniformly radiolabeled, single-stranded *mtrA* probes synthesized using primer *mtrA*S5 (I) or *mtrA*S6 (II) ending at the *Age*I site. *mtrA*S6 contained a 10-bp overhang of an irrelevant sequence at the 5′ end which did not correspond to *mtrA*. Lanes: 1, S1 digestion; 2, probe loaded in amounts diluted 100× relative to lane 1. The bent arrow indicates the location of the translational start site (+1). Note that S1 nuclease products in panel A coincide with +1 while in B they are at +10 due to the 5′ overhang of probe II that is not complementary to *mtrA* mRNA. (C) Primer extension analysis. Reverse transcription was performed using 10 μg of total cellular RNA from *M. tuberculosis* H37Rv and end-labeled primer *mtrA*S8 (extension product III). Lanes: 1, reaction mixture containing RNA; 2, control reaction mixture without RNA. (D) Schematic representation of S1 nuclease probes (I and II), reverse transcription products (III), and the primers (*mtrA*S5, -S6, and -S8) used to generate them.

macrophages over a 5-day period (Fig. 3E to G). As a control, the previously characterized *hsp60-gfp* fusion (7) was fluorescent in both *M. bovis* BCG and *M. tuberculosis* H37Rv during macrophage infection (Fig. 3D and H and data not shown). Next we tested the expression of the *mtrA-gfp* fusion in *M. tuberculosis* H37Rv cells in human macrophages derived from peripheral blood monocytes. These experiments showed similar results (Fig. 3I to L). A quantitative analysis of fluorescence in J774 cells is shown in Fig. 4. Different expression in *M. bovis* BCG and *M. tuberculosis* H37Rv has also been observed with other two-component response regulators from *M. tuberculosis* (T.C.Z. and V.D., unpublished results). In conclusion, *mtrA*

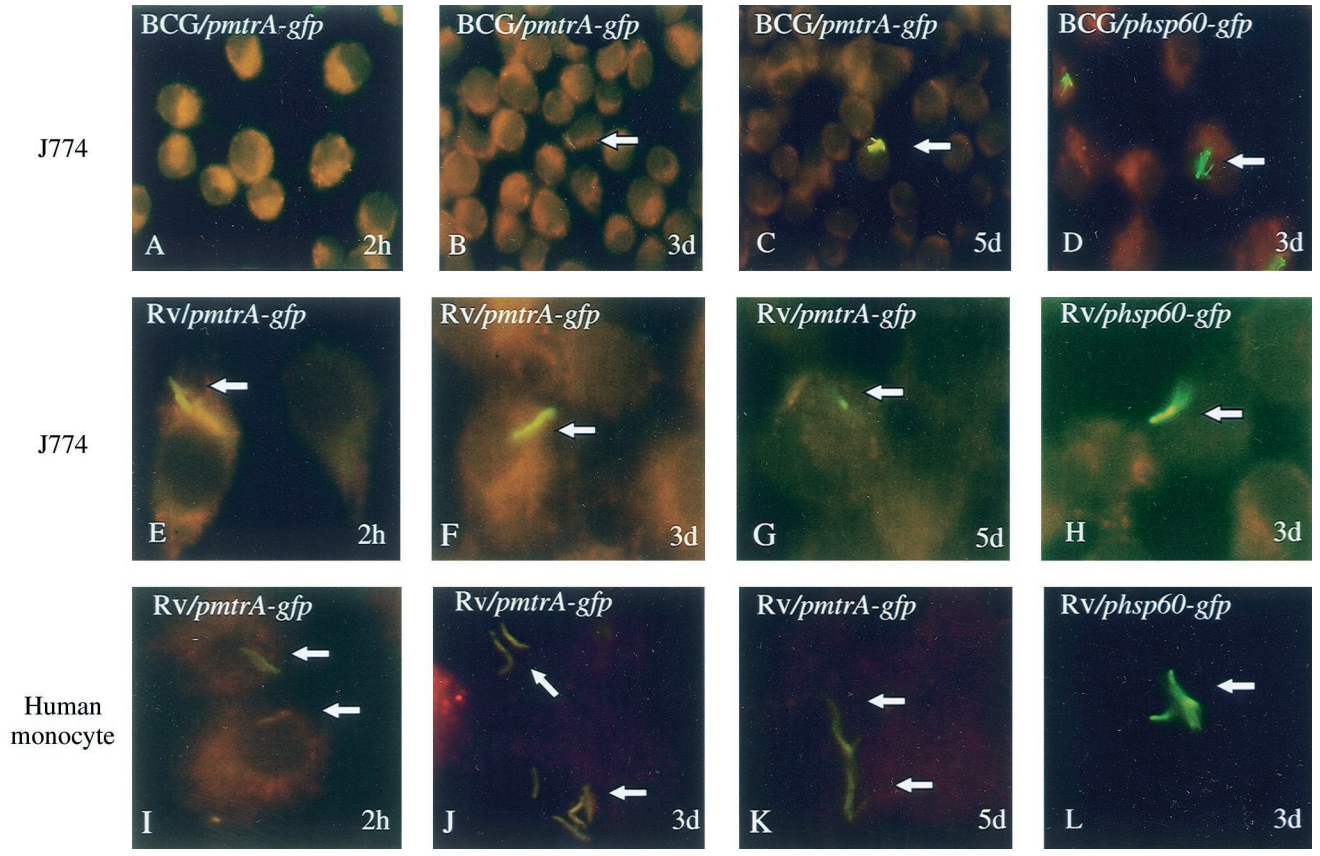

FIG. 3. Expression of *mtrA* in infected macrophages monitored by *mtrA-gfp* fusion and epifluorescence microscopy. *M. tuberculosis* H37Rv or *M. bovis* BCG carrying the p*mtrA-gfp* or p*hsp60-gfp* reporter plasmid was used to infect macrophages, which were incubated for 2 h, 3 days, or 5 days before the cells were fixed and prepared for fluorescence microscopy analysis. Panels: A to H, J774 murine macrophage-like cell line; I to L, human peripheral blood monocyte-derived macrophages. Arrows point at the bacteria within the macrophages.

is expressed during in vitro growth in virulent *M. tuberculosis* and its expression differs significantly between *M. tuberculosis* H37Rv and the vaccine strain *M. bovis* BCG during growth in macrophages.

Essential two-component systems in bacteria. To our knowledge, this is the first report of an essential two-component signal transduction system in *M. tuberculosis*. Although rare, essential two-component systems have been reported in other bacterial species. For example, *Caulobacter crescentus* encodes two essential two-component systems, CtrA-CckA (17, 29) and DivK-DivJ (15, 36), that are required for cell cycle regulation in this organism. The CtrA-CckA system has been shown to regulate genes involved in at least five distinct cell cycle events, including flagellar biogenesis, DNA methylation, and DNA replication (29). The CtrA response regulator also controls the differentiation of the swarmer cell type into the stalked cell type by directly binding to sites present within the chromosomal origin of replication, thus blocking an essential DnaA box and promoter necessary for replication initiation (29). Interestingly, the other essential two-component system, DivK-DivJ, appears to mediate cell cycle regulation through the CtrA-CckA system, adding further complexity to this already multicomponent regulatory system (17, 36). In *Bacillus subtilis*, an essential two-component signal transduction system, *yycF-yycG*, has also been described (9, 10); however, the process(es) regulated by this system remains unknown. In addition, essential two-component systems have also been identified in pathogenic organisms, as an essential two-component system from

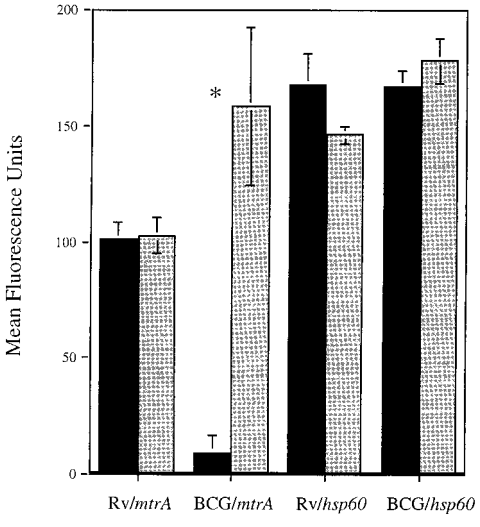

FIG. 4. Quantitation of fluorescence intensities of *mtrA-gfp* and *hsp60-gfp* fusions in *M. tuberculosis* H37Rv and *M. bovis* BCG infecting J774 cells. Mean fluorescence intensities (± the standard errors) were determined as described in Materials and Methods. Macrophages were infected for 2 h (black bars) or 3 days (grey bars) with *M. tuberculosis* H37Rv or *M. bovis* BCG Pasteur carrying the p*mtrA-gfp* or p*hsp60-gfp* reporter plasmid. *, significant difference in fluorescence intensity ($P < 0.05$ by ANOVA).

Staphylococcus aureus showing high similarity to *yycF-yycG* from *B. subtilis* has recently been reported (23). Initial characterization of this system in *S. aureus* suggests that its role includes the proper regulation of bacterial cell wall or membrane composition (23). In addition, among the 13 two-component signal transduction systems present in *Streptococcus pneumoniae*, one two-component response regulator could not be inactivated (20, 34), suggesting that this system is also required for an essential cellular function.

Although *mtrB* is located immediately downstream of *mtrA* and probably encodes its cognate sensor histidine kinase (35), our results suggest that *mtrB* is not essential for the growth of *M. tuberculosis* in vitro. The identification of a nonessential histidine kinase closely linked to an essential response regulator has also been observed in *S. pneumoniae* (34); however, other essential two-component systems appear to require both a response regulator and its cognate histidine kinase for growth in vitro (9, 10, 17, 23, 29). It is not known whether *mtrA* is also essential in *M. bovis* BCG. As low-level expression of *mtrA* may be sufficient to exert its function, we anticipate that *mtrA* could be essential for the growth of *M. bovis* BCG in vitro. Regardless, the presence of an essential two-component signal transduction system in *M. tuberculosis* underscores the need for further characterization of the functions controlled by this and other regulators of this type in the tubercle bacillus.

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REFERENCES

1. **Aldovini, A., R. N. Husson, and R. A. Young.** 1993. The *uraA* locus and homologous recombination in *Mycobacterium bovis* BCG. J. Bacteriol. **175:** 7282–7289.
2. **Bloom, B. R., and C. J. Murray.** 1992. Tuberculosis: commentary on a reemergent killer. Science **257:**1055–1064.
3. **Boyum, A.** 1968. Separation of leukocytes from blood and bone marrow. Introduction. Scand. J. Clin. Lab. Investig. Suppl. **97:**7.
4. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, et al.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537–544.
5. **Curcic, R., S. Dhandayuthapani, and V. Deretic.** 1994. Gene expression in mycobacteria: transcriptional fusions based on *xylE* and analysis of the promoter region of the response regulator *mtrA* from *Mycobacterium tuberculosis*. Mol. Microbiol. **13:**1057–1064.
6. **Dhandayuthapani, S., M. Mudd, and V. Deretic.** 1997. Interactions of OxyR with the promoter region of the *oxyR* and *ahpC* genes from *Mycobacterium leprae* and *Mycobacterium tuberculosis*. J. Bacteriol. **179:**2401–2409.
7. **Dhandayuthapani, S., L. E. Via, C. A. Thomas, P. M. Horowitz, D. Deretic, and V. Deretic.** 1995. Green fluorescent protein as a marker for gene expression and cell biology of mycobacterial interactions with macrophages. Mol. Microbiol. **17:**901–912.
8. **Dhandayuthapani, S., Y. Zhang, M. H. Mudd, and V. Deretic.** 1996. Oxidative stress response and its role in sensitivity to isoniazid in mycobacteria: characterization and inducibility of *ahpC* by peroxides in *Mycobacterium smegmatis* and lack of expression in *M. aurum* and *M. tuberculosis*. J. Bacteriol. **178:**3641–3649.
9. **Fabret, C., V. A. Feher, and J. A. Hoch.** 1999. Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. J. Bacteriol. **181:** 1975–1983.
10. **Fabret, C., and J. A. Hoch.** 1998. A two-component signal transduction system essential for growth of *Bacillus subtilis*: implications for anti-infective therapy. J. Bacteriol. **180:**6375–6383.
11. **Garbe, T. R., J. Barathi, S. Barnini, Y. Zhang, C. Abou-Zeid, D. Tang, et al.** 1994. Transformation of mycobacterial species using hygromycin resistance as selectable marker. Microbiology **140:**133–138.
12. **Graham, J. E., and J. E. Clark-Curtiss.** 1999. Identification of *Mycobacterium tuberculosis* RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). Proc. Natl. Acad. Sci. USA **96:**11554–11559.
13. **Guilhot, C., B. Gicquel, and C. Martin.** 1992. Temperature-sensitive mutants of the Mycobacterium plasmid pAL5000. FEMS Microbiol. Lett. **77:**181–186.
14. **Haydel, S. E., N. E. Dunlap, and W. H. Benjamin, Jr.** 1999. *In vitro* evidence of two-component system phosphorylation between the *Mycobacterium tuberculosis* TrcR/TrcS proteins. Microb. Pathog. **26:**195–206.
15. **Hecht, G. B., T. Lane, N. Ohta, J. M. Sommer, and A. Newton.** 1995. An essential single domain response regulator required for normal cell division and differentiation in *Caulobacter crescentus*. EMBO J. **14:**3915–3924.
16. **Hoch, J. A., and T. J. Silhavy (ed.).** 1995. Two-component signal transduction. American Society for Microbiology, Washington, D.C.
17. **Jacobs, C., I. J. Domian, J. R. Maddock, and L. Shapiro.** 1999. Cell cycle-dependent polar localization of an essential bacterial histidine kinase that controls DNA replication and cell division. Cell **97:**111–120.
18. **Jacobs, W. R., Jr., G. V. Kalpana, J. D. Cirillo, L. Pascopella, S. B. Snapper, R. A. Udani, et al.** 1991. Genetic systems for mycobacteria. Methods Enzymol. **204:**537–555.
19. **Kalpana, G. V., B. R. Bloom, and W. R. Jacobs, Jr.** 1991. Insertional mutagenesis and illegitimate recombination in mycobacteria. Proc. Natl. Acad. Sci. USA **88:**5433–5437.
20. **Lange, R., C. Wagner, A. de Saizieu, N. Flint, J. Molnos, M. Stieger, et al.** 1999. Domain organization and molecular characterization of 13 two-component systems identified by genome sequencing of *Streptococcus pneumoniae*. Gene **237:**223–234.
21. **Magdalena, J., P. Supply, and C. Locht.** 1998. Specific differentiation between *Mycobacterium bovis* BCG and virulent strains of the *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. **36:**2471–2476.
22. **Magdalena, J., A. Vachée, P. Supply, and C. Locht.** 1998. Identification of a new DNA region specific for members of *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. **36:**937–943.
23. **Martin, P. K., T. Li, D. Sun, D. P. Biek, and M. B. Schmid.** 1999. Role in cell permeability of an essential two-component system in *Staphylococcus aureus*. J. Bacteriol. **181:**3666–3673.
24. **Misra, T. K.** 1987. DNA sequencing: a new strategy to create ordered deletions, modified M13 vector, and improved reaction conditions for sequencing by dideoxy chain termination method. Methods Enzymol. **155:** 119–139.
25. **Mohr, C. D., D. W. Martin, W. M. Konyecsni, J. R. Govan, S. Lory, and V. Deretic.** 1990. Role of the far-upstream sites of the *algD* promoter and the *algR* and *rpoN* genes in environmental modulation of mucoidy in *Pseudomonas aeruginosa*. J. Bacteriol. **172:**6576–6580.
26. **Pagan-Ramos, E., J. Song, M. McFalone, M. H. Mudd, and V. Deretic.** 1998. Oxidative stress response and characterization of the *oxyR-ahpC* and *furA-katG* loci in *Mycobacterium marinum*. J. Bacteriol. **180:**4856–4864.
27. **Parrish, N. M., J. D. Dick, and W. R. Bishai.** 1998. Mechanisms of latency in *Mycobacterium tuberculosis*. Trends Microbiol. **6:**107–112.
28. **Pelicic, V., J. M. Reyrat, and B. Gicquel.** 1996. Expression of the *Bacillus subtilis sacB* gene confers sucrose sensitivity on mycobacteria. J. Bacteriol. **178:**1197–1199.
29. **Quon, K. C., G. T. Marczynski, and L. Shapiro.** 1996. Cell cycle control by an essential bacterial two-component signal transduction protein. Cell **84:** 83–93.
30. **Rappuoli, R., V. Scarlato, and B. Arico (ed.).** 1995. Signal transduction and bacterial virulence. R. G. Landes Company, Austin, Tex.
31. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, et al.** 1991. New use of BCG for recombinant vaccines. Nature **351:** 456–460.
32. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res. **20:**961–974.
33. **Supply, P., J. Magdalena, S. Himpens, and C. Locht.** 1997. Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. Mol. Microbiol. **26:**991–1003.
34. **Throup, J. P., K. K. Koretke, A. P. Bryant, K. A. Ingraham, A. F. Chalker, Y. Ge, et al.** 2000. A genomic analysis of two-component signal transduction in *Streptococcus pneumoniae*. Mol. Microbiol. **35:**566–576.
35. **Via, L. E., R. Curcic, M. H. Mudd, S. Dhandayuthapani, R. J. Ulmer, and V. Deretic.** 1996. Elements of signal transduction in *Mycobacterium tuberculosis*: in vitro phosphorylation and in vivo expression of the response regulator MtrA. J. Bacteriol. **178:**3314–3321.
36. **Wu, J., N. Ohta, and A. Newton.** 1998. An essential, multicomponent signal transduction pathway required for cell cycle regulation in *Caulobacter*. Proc. Natl. Acad. Sci. USA **95:**1443–1448.