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The *prrAB* Two-Component System Is Essential for *Mycobacterium tuberculosis* Viability and Is Induced under Nitrogen-Limiting Conditions

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The *Mycobacterium tuberculosis prrA-prrB* (Rv0903c-Rv0902c) two-component regulatory system is expressed during intracellular growth in human macrophages and is required for early intracellular multiplication in murine macrophages, suggesting its importance in establishing infection. To better understand the function of the *prrA-prrB* two-component system, we defined the transcriptional characteristics of the *prrA* and *prrB* genes during exponential and stationary growth and upon exposure to different environmental stresses and attempted to generate a *prrA-prrB* deletion mutant. The *prrA* and *prrB* genes constitute an operon and are cotranscribed during logarithmic growth, with transcriptional levels decreasing in stationary phase and during hypoxia. Despite the transcriptional differences, PrrA protein levels remained relatively stable throughout growth and in hypoxia. Under conditions of nitrogen limitation, *prrAB* transcription was induced, while acidic pH stress and carbon starvation did not significantly alter transcript levels. Deletion of the *prrAB* operon on the chromosome of *M. tuberculosis* H37Rv occurred only in the presence of an episomal copy of the *prrAB* genes, indicating that this two-component system is essential for viability. Characterization of the *prrAB* locus in *M. tuberculosis* Mt21D3, a previously described *prrA* transposon mutant, revealed that this strain is not a true *prrA* knockout mutant. Rather, Tn*5367* transposon insertion into the *prrA* promoter only decreased *prrA* and *prrB* transcription and PrrA levels in Mt21D3 compared to those in the parental Mt103 clinical strain. These data provide the first report describing the essentiality of the *M. tuberculosis prrAB* two-component system and reveal insights into its potential role in mycobacterial growth and metabolism.

Tuberculosis continues to be a global health emergency. According to 2009 WHO statistics, 9.4 million new cases of tuberculosis were diagnosed and 1.7 million people died from the disease (equivalent to 4,700 deaths each day) (29). This global emergency is further exacerbated by multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis* strains that are resistant to our best antibiotics and thus difficult to treat (14). A hallmark in the life cycle of *M. tuberculosis* is its intracellular residence within the human macrophage. To ensure its intracellular survival, *M. tuberculosis* must adapt to the host environment by appropriately regulating the expression of genes involved in virulence and metabolism. Understanding how *M. tuberculosis* regulatory systems coordinate complex adaptations is critical to deciphering the ongoing interactions that govern establishment and progression of tuberculosis disease.

Like most bacteria, *M. tuberculosis* uses two-component systems to execute transcriptional reprogramming in response to changing environments. Of the 11 paired two-component systems, two orphan histidine kinase genes, and six orphan response regulator genes (15), at least three *M. tuberculosis* response regulators (*phoP*, *devR*, and *mprA*) have been implicated in mycobacterial virulence (6, 19, 23) and persistence (30, 32). However, thus far, only the *M. tuberculosis mtrA* response regulator gene has been deemed essential for viability (31). Although *mtrA* and *mtrB* (histidine kinase gene) are genetically linked on the chromosome, *mtrB* was not essential for *M. tuberculosis* growth *in vitro* (31).

The *prrAB* two-component regulatory system has been shown to be expressed during growth in human macrophages and is required for early intracellular multiplication (7, 12, 15). Moreover, the *prrAB* regulatory system is one of only four two-component systems conserved in all mycobacterial species (28), thus strongly suggesting its fundamental importance in mycobacteria. Based on these data and observations, we hypothesized that the *prrAB* two-component system was critical for *M. tuberculosis* survival or virulence within the host. In this study, we have begun delineating *prrAB* expression characteristics and have determined that the *prrAB* signal transduction system is essential for *M. tuberculosis* viability and may play a role in regulating gene expression during nitrogen-limiting conditions.

MATERIALS AND METHODS

Bacterial media and culture conditions. *Escherichia coli* JM109 was grown in Luria-Bertani (LB) broth or on LB agar plates at 37°C with hygromycin (Hyg) (150 μg/ml) added as necessary. *M. tuberculosis* H37Rv cultures were grown at 37°C in Middlebrook 7H9 basal liquid medium (Difco) supplemented with 10% ADS (0.5% bovine serum albumin fraction V, 0.2% dextrose, 140 mM NaCl) enrichment and 0.05% Tween 80 (herein described as supplemented Middlebrook 7H9) with or without 0.2% glycerol and Hyg (100 μg/ml) when required. *M. tuberculosis* H37Rv was cultivated on Middlebrook 7H9, 7H10, or 7H11 agar (Difco) supplemented with 10% ADS enrichment with or without 0.2% glycerol and various concentrations of Hyg (described below). *M. tuberculosis* Mt103 and Mt21D3 strains were grown similarly to H37Rv, with

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kanamycin (Km) (25 μg/ml) included for Mt21D3 growth. *Mycobacterium smegmatis* mc²155 was grown at 37°C in Middlebrook 7H9 liquid medium or LB broth supplemented with 0.5% Tween 80 or on LB agar plates. During *M. tuberculosis* H37Rv *prrAB* mutagenesis attempts, additional medium supplements, varied post-mycobacteriophage infection outgrowth times, and modifications of Middlebrook 7H9, 7H10, or 7H11 agar with various concentrations of Hyg were as follows: (i) medium supplements, Casamino Acids (0.2% or 0.4%), Casitone (0.1%), and tryptophan (20 or 40 μg/ml); (ii) outgrowth times, 24 h and 72 h; (iii) Hyg concentrations, 50, 75, 100, 125, or 150 μg/ml.

Exposure of *M. tuberculosis* to environmental stresses. *M. tuberculosis* H37Rv cultures (optical density at 600 nm [OD_{600}] of ~0.2 to 0.4) were harvested, washed with Middlebrook 7H9 basal medium (minus ADS supplement), and resuspended in stress-specific medium. For acid stress, the cultures were resuspended in Middlebrook 7H9-Tween 80-ADS medium with the pH adjusted to 5.5. For carbon stress, cultures were resuspended in Middlebook 7H9-Tween 80-albumin-NaCl medium (without dextrose or glycerol). To generate nitrogen-limiting conditions, the Amon et al. (2) model, whereby cells are incubated in Middlebrook 7H9-Tween 80-ADS medium supplemented with 200 μM L-methionine *S*-sulfoximine (MSX), was employed. Treated and untreated control cultures were grown aerobically for 4 h and 4 days, followed by RNA isolation as described below. For hypoxia, *M. tuberculosis* cultures were grown in Dubos-Tween 80-albumin broth to an OD_{600} of ~0.6 and subjected to hypoxia in standing, sealed tubes for 24 and 48 h as described previously (24).

RNA isolation. RNA isolation was performed using TRI Reagent solution (Applied Biosystems/Ambion, Austin, TX) according to the manufacturer's protocol and as described previously (20). Briefly, cells were lysed mechanically using a FastPrep bead beater (Qbiogene/MP Biomedicals) with three pulses of 30 s each at 6,500 rpm in a mixture of 0.1 mm zirconium-silica beads and 1 ml of TRI Reagent solution in a 2-ml sterile screw-cap tube. The RNA in the aqueous phase was extracted with chloroform, precipitated using isopropanol, and treated with RNase-free Turbo DNase (Applied Biosystems/Ambion) for 1 h at 37°C to remove any DNA contamination. The DNase-treated RNA was then determined to be free of DNA contamination by confirming the lack of 16S rRNA gene amplification. The RNA concentration was determined using a NanoDrop 1000 spectrophotometer (NanoDrop/Thermo Scientific), and the quality of the RNA was assessed using Experion, an automated capillary electrophoresis system (Bio-Rad Laboratories).

qRT-PCR. For quantitative reverse transcription-PCR (qRT-PCR), 100 ng of RNA was converted to cDNA by reverse transcription using the iScript cDNA synthesis kit (Bio-Rad Laboratories). To further verify the absence of contaminating genomic DNA, RNA samples were concurrently analyzed using RT-PCR without reverse transcriptase. The cDNA was diluted (1:10) and used as a template for quantitative PCR (qPCR) using gene-specific primers and SYBR green dye. Optimization of the PCR amplification parameters was performed using iQ SYBR green supermix (Bio-Rad Laboratories). Standard curve generation and melting-curve analyses were performed as described previously (20). For each qPCR experiment, the calculated threshold cycle (C_T) was normalized to the C_T of the internal 16S rRNA control (amplified from the same samples) before calculating the fold change between the test and control samples.

Northern analyses. Antisense *prrA* and *prrB* RNA digoxigenin (DIG)-labeled probes were prepared using the DIG Northern starter kit (Roche, Indianapolis, IN), following the manufacturer's protocol. One microgram of total *M. tuberculosis* H37Rv RNA was isolated from logarithmic-phase cells, separated via a 1% agarose-glyoxyl gel, and transferred to a BrightStar membrane (Applied Biosystems/Ambion) using NorthernMax kit reagents (Applied Biosystems/Ambion). The transferred RNA was hybridized with the gene-specific, denatured DIG-labeled RNA probe and detected using the NorthernMax protocol (Applied Biosystems/Ambion). To ensure complete stripping of the DIG-labeled probe before subsequent hybridizations, membranes were incubated twice in a 50% formamide solution containing 50 mM Tris-HCl, pH 7.5, and 5% SDS at 80°C for 60 min and then subjected to autoradiography.

Mycobacterial protein extracts. *M. tuberculosis* cells were washed twice in wash buffer (phosphate-buffered saline [PBS] buffer containing protease inhibitor cocktail [Roche], 1 mM dithiothreitol [DTT], 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and lysed in wash buffer or two-dimensional (2D)-rehydration buffer (Bio-Rad Laboratories) with 0.1-mm-diameter zirconia/silica beads (Biospec Products) in a Fast Prep homogenizer. Lysed cells were centrifuged at 12,000 rpm at 4°C for 10 min, and the supernatant containing the cell-free protein extract was collected. Protein concentrations were determined using the RC-DC protein assay kit (Bio-Rad Laboratories) with bovine serum albumin as a standard.

PrrA antibody generation and Western analyses. The recombinant 6×-His-tagged PrrA protein was produced in *E. coli* and purified as previously described (17). The purified PrrA protein (~100 μg) was emulsified in Freund's incomplete adjuvant and used to immunize two New Zealand White rabbits by subcutaneous injection. Rabbits were given booster injections of PrrA antigen at 4-week intervals, and antisera were collected after at least two booster injections. After protein quantitation, the samples were resolved via SDS-PAGE, transferred onto a nitrocellulose membrane, and stained with Ponceau S stain to ensure equivalent amounts of transferred protein. Immunoblotting was performed using rabbit anti-PrrA rabbit polyclonal antiserum (1:20,000) and detected with the Supersignal West Dura chemiluminescent substrate (Thermo Scientific). Quantitations were performed using the ImageJ 1.44 densitometry software program (NIH).

Generation of a Δ*prrAB*::*hyg* thermosensitive mycobacteriophage and mutagenesis procedures. The Δ*prrAB*::*hyg* allele was constructed by amplifying the flanking regions of the *prrAB* genes and cloning the fragments into pYUB854 (4) on either side of the *hyg* resistance cassette. The 1,000-bp region upstream of the *prrAB* genes was amplified using the primers SH81 (5′-CCCAAGCTTGATGCTGGATCGTGCGTTGAC-3′) and SH82 (5′-CTAGCTAGCTTTGCCTGATTACCGTCCAGC-3′), which contain HindIII and NheI sites (underlined) at their respective 5′ termini. The 989-bp region downstream of the *prrAB* genes was amplified using the primers SH83 (5′-TGCTCTAGAGGCACCGCGTCGCTGGAGAA-3′) and SH84 (5′-CGGGGTACCACCGGCAGCGAAGGTATCAA-3′), which contain XbaI and KpnI sites (underlined) at their respective 5′ termini. The PCR products were cloned into pYUB854 (4), flanking the *hyg* cassette, to create pSH270. The ligation mixture of PacI-digested pSH270 and PacI-digested concatamerized phAE87 DNA was packaged using Gigapack III packaging extracts (Stratagene) and transduced into *E. coli* HB101. Phasmid DNA was prepared from pooled Hyg-resistant (Hyg^r) transductants, digested with appropriate restriction enzymes to verify the presence of the desired insert, and electroporated into *M. smegmatis* mc²155. Transformants were plated and incubated at 30°C for the generation of mycobacteriophage plaques. A high-titer mycobacteriophage stock was generated from a confirmed temperature-sensitive phage (phSH270) and was used to infect *M. tuberculosis* H37Rv as previously described (4, 16).

The *prrAB*-complementing plasmid, pSH439, consists of 125 bp upstream of *prrA*, the *prrAB* coding regions, and 15 bp downstream of the *prrB* stop codon cloned into pMV306, a site-specific (*att*) integrating mycobacterial vector (26). Confirmed integration of pSH439 into *M. tuberculosis* H37Rv generated *M. tuberculosis* STS16, which harbored an ectopic *prrAB* locus. The phSH270 Δ*prrAB*::*hyg* thermosensitive mycobacteriophage was used to infect *M. tuberculosis* STS16 as previously described (4, 16), and Hyg- and Km-resistant colonies were screened by PCR or Southern blot analysis for deletion of the wild-type *prrAB* locus. Two confirmed *prrAB*-complemented Δ*prrAB*::*hyg* deletion mutants were isolated and designated *M. tuberculosis* STS22 and STS23.

Southern blot analyses. Genomic DNA was isolated via enzymatic lysis from *M. tuberculosis* cells harvested in the mid-logarithmic phase of growth, digested with the appropriate restriction enzymes, separated by

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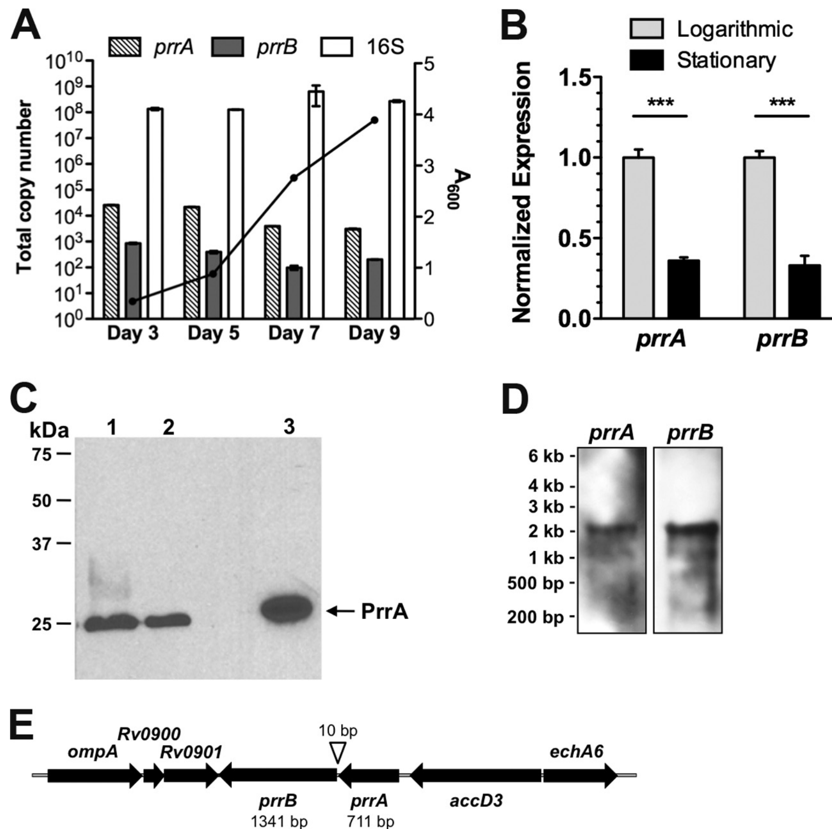

FIG 1 *prrA* and *prrB* transcription and PrrA levels during *in vitro* growth. (A) Quantitation of *prrA* and *prrB* transcripts during *M. tuberculosis* H37Rv *in vitro* exponential growth in supplemented Middlebrook 7H9 broth with glycerol. The optical density at 600 nm of the cultures is indicated by the solid line and scaled on the right *y* axis. (B) qRT-PCR comparison of *prrA* and *prrB* expression in logarithmic and stationary phases of *M. tuberculosis* growth in supplemented Middlebrook 7H9 broth without glycerol. Normalized expression of *prrA* and *prrB* with respect to 16S rRNA expression is presented as the mean ± SD of data from three independent experiments. The decrease in *prrA* and *prrB* expression during stationary phase is presented relative to the logarithmic-phase expression, which is set at 1.0. ***, $P < 0.0001$. (C) Western blot analyses of PrrA in total *M. tuberculosis* H37Rv protein (20 μg) collected from logarithmic (lane 1) and stationary (lane 2) growth phases and detected using anti-PrrA antibodies. Lane 3 represents the purified His-tagged PrrA protein (40 ng). (D) Northern analysis of *M. tuberculosis* H37Rv with *prrA* and *prrB* single-stranded RNA probes. RNA samples were collected from exponential-phase cultures of *M. tuberculosis* H37Rv grown in Middlebrook 7H9 broth with glycerol. (E) Diagram of the *prrA-prrB* region of the chromosome, drawn to scale and showing the *prrA* and *prrB* gene lengths.

agarose gel electrophoresis, and transferred to a positively charged nylon membrane. Transferred DNAs were then hybridized with the gene-specific DIG-labeled probes and detected using anti-DIG-alkaline phosphatase antibody and the CDP-Star chemiluminescent reagent as recommended by the manufacturer (Roche). Membranes were then exposed to X-ray film at room temperature.

Statistical analyses. Statistical analyses were performed using the Prism 5 software program (GraphPad Software, San Diego, CA) and were calculated using a Student *t* test or analysis of variance (ANOVA). A *P* value of <0.05 was considered statistically significant.

RESULTS

Characterization of *prrA* and *prrB* transcription during *in vitro* growth. Since the *prrAB* two-component system is evolutionarily conserved among all mycobacterial genomes and plays a role in early stages of *M. tuberculosis* infection, we investigated its physiological characteristics. Compared to 16S rRNA levels, transcription of *prrA* and *prrB* is generally low throughout exponential growth of *M. tuberculosis* H37Rv *in vitro*, with transcriptional levels of *prrA* consistently more abundant than those of *prrB* (Fig. 1A). Transcription of *prrA* and *prrB* was greatest during early logarithmic growth and was slightly decreased as the culture progressed into late exponential growth (Fig. 1A). These *prrA* and *prrB* transcriptional trends during exponential growth were similar during *M. tuberculosis* H37Rv growth in the absence of glycerol (data not shown).

The PrrA protein is stable in stationary phase. To investigate *prrA* and *prrB* transcriptional trends throughout growth, we compared *prrA* and *prrB* levels during *M. tuberculosis* H37Rv logarithmic (3 days) and stationary (30 days) phases of growth. After 30 days of growth in supplemented Middlebrook 7H9 without glycerol, representing stationary phase, *prrA* and *prrB* transcription was decreased by 60% compared to expression during logarithmic growth (3 days) (Fig. 1B). However, Western blot analysis of total protein isolated at the above-specified times, followed by densitometry quantitation, revealed that production of PrrA in stationary-phase cultures was slightly decreased, by ~20%, from that in logarithmic-phase cultures (Fig. 1C and Fig. 2C, lanes 4 and 5). The levels of PrrA in stationary phase did not correlate with the decreased transcript level (Fig. 1B), indicating that the PrrA protein is relatively stable in stationary phase.

***prrA* and *prrB* are cotranscribed in an operon.** To determine if the transcriptional differences were due to the presence of inde-

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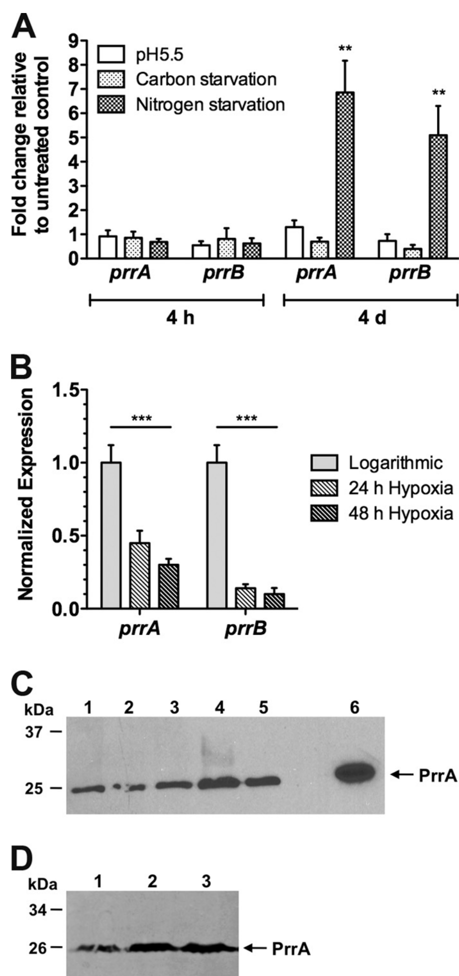

FIG 2 *M. tuberculosis prrA* and *prrB* are differentially transcribed and translated upon exposure to *in vitro* environmental stress conditions. (A) qRT-PCR comparison of *prrA* and *prrB* expression upon *M. tuberculosis* H37Rv exposure to low pH (pH 5.5), carbon starvation, or nitrogen-limiting environments. The fold change in *prrA* and *prrB* expression in H37Rv grown under environmental stress conditions with respect to H37Rv grown in Middlebrook 7H9-Tween-ADS medium (untreated control) (baseline expression set to 1.0) is presented as the mean ± SD of data from three independent experiments. **, *prrA P* value = 0.0013 or *prrB P* value = 0.0032. d, days. (B) qRT-PCR analysis of *prrA* and *prrB* expression, comparing logarithmic growth and 24- or 48-h exposure to hypoxic conditions. Normalized expression of *prrA* and *prrB* with respect to 16S rRNA expression is presented as the mean ± SD of data from three independent experiments. The decrease in *prrAB* transcript levels in hypoxia is presented relative to logarithmic-phase expression, which is set at 1.0. ***, *prrA P* value = 0.0002 by ANOVA or *prrB P* value < 0.0001 by ANOVA. (C) Western blot analysis of PrrA during *M. tuberculosis* H37Rv logarithmic growth, stationary phase, and hypoxia. Total protein (40 μg) was separated via SDS-PAGE and detected using polyclonal anti-PrrA antibodies with purified His-tagged PrrA protein (40 ng) serving as a positive control. Lanes: 1, aerobic growth control; 2, hypoxia, 24 h; 3, hypoxia, 48 h; 4, logarithmic growth, 3 days; 5, stationary phase, 30 days; 6, purified PrrA protein. (D) Western blot analysis of PrrA during *M. tuberculosis* H37Rv nitrogen limitation, logarithmic growth, stationary phase, and hypoxia. Total protein (40 μg) was separated via SDS-PAGE and detected using polyclonal anti-PrrA antibodies, with the purified His-tagged PrrA protein (20 ng) serving as a positive control. Lanes: 1, MSX exposure, 4 h; 2, MSX exposure, 4 days; 3, purified PrrA protein.

pendent *prrA* and *prrB* expression, we used Northern blot analyses to establish the length of the *prrA* and *prrB* transcripts. Northern analyses using DIG-labeled *prrA* and *prrB* single-stranded RNA probes revealed the presence of a single transcript of approximately 2.1 kb (Fig. 1D). Repeated hybridizations did not reveal the presence of smaller transcripts. Therefore, we concluded that in *M. tuberculosis* H37Rv, *prrA* and *prrB*, like most genetically linked two-component systems, are cotranscribed under the growth conditions tested in an apparent operon with a 10-bp intergenic region separating *prrA* and *prrB* (Fig. 1E).

***prrA* and *prrB* transcription is induced under nitrogen limitation and repressed in hypoxia.** To begin characterizing environmental conditions that could stimulate PrrAB activity, we analyzed *prrA* and *prrB* transcript levels in *M. tuberculosis* H37Rv upon short (4 h) and prolonged (4 days) exposure to nitrogen-limiting conditions, acid stress, carbon starvation, and hypoxia. Nitrogen limitation was induced by exposure to MSX, which is known to inhibit *M. tuberculosis* glutamine synthetase GlnA1, a crucial enzyme in nitrogen metabolism and virulence (13, 21, 27). Based on qRT-PCR analyses, *prrA* and *prrB* were induced 7- and 5-fold, respectively, when *M. tuberculosis* was incubated for 4 days under nitrogen-limiting conditions (Fig. 2A). No induction was observed in MSX-treated samples at 4 h postexposure. *prrA* and *prrB* transcriptional changes upon exposure to a low-pH environment and during carbon starvation were not statistically significant (Fig. 2A). Incubation of *M. tuberculosis* under hypoxic conditions for 24 h or 48 h resulted in significantly decreased transcription of both *prrA* and *prrB* (Fig. 2B). However, despite *prrA* transcriptional changes (Fig. 2B), PrrA levels were similar and stable in hypoxia (Fig. 2C, lanes 2 and 3) compared to those of the aerobic control culture (Fig. 2C, lane 1). Most importantly, comparison of MSX-treated cultures revealed that after 4 days of MSX exposure, PrrA levels were increased by 89% compared to results with 4 h of MSX exposure (Fig. 2D), thus corroborating the induction observed at the transcription level (Fig. 2A). Together, these results suggest that the PrrAB two-component system is capable of responding to environmental changes and may be required in stress-induced metabolic and physiological adaptation of *M. tuberculosis*, specifically adaptation to nitrogen limitation.

***prrAB* is an essential two-component system.** Since the *prrAB* system is significantly induced under nitrogen-limiting conditions (Fig. 2A), expressed after 48 h of intracellular growth in human macrophages, and required for early intracellular multiplication in murine bone marrow-derived macrophages (7, 12, 15), we attempted to delete the *M. tuberculosis prrAB* two-component system using the thermosensitive mycobacteriophage mutagenesis strategy, in which a hygromycin resistance cassette replaced the entire *prrA* open reading frame (ORF) and 99% of the *prrB* ORF. A total of 11 independent *M. tuberculosis* H37Rv Δ*prrAB*::*hyg* thermosensitive mycobacteriophage infections were performed, yielding 1,325 Hyg-resistant (Hyg^r) CFU, of which 231 independent cultures were grown and screened by PCR or Southern blot analysis for the presence of the Δ*prrAB*::*hyg* mutation. After screening, none had undergone allelic exchange to yield *prrAB* deletion mutants. In an attempt to recover an *M. tuberculosis* Δ*prrAB*::*hyg* mutant, we also supplemented either Middlebrook 7H9, 7H10, or 7H11 agar with various concentrations of Hyg, Casamino Acids, Casitone, and/or tryptophan and varied post-mycobacteriophage infection outgrowth times (see Materials and Methods). The inability to delete the *prrAB* genes from *M. tuberculosis* after 11 successive, independent phage mutagenesis attempts, while incorporating various supplementation and outgrowth approaches, strongly suggested that the *prrAB* two-component system is essential for viability under the de-

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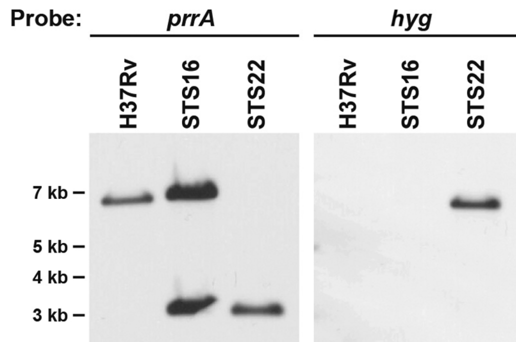

FIG 3 Southern blot analysis of *M. tuberculosis* chromosomal DNA from wild-type H37Rv, STS16 (H37Rv::*prrAB*), and STS22 (*prrAB*-complemented Δ*prrAB*::*hyg* deletion mutant), confirming generation of a wild-type Δ*prrAB*::*hyg* mutation in the presence of an ectopic copy of *prrAB*. Genomic DNA was digested with HpaI and SpeI and hybridized with the *prrA* gene and the hygromycin (*hyg*) resistance cassette. Expected sizes of the hybridized DNA fragments: wild-type *prrA*, 6,823 bp; episomal copy of *prrA*, 3,048 bp; *hyg*, 6,730 bp. Molecular size markers (in kilobases) are shown on the left.

scribed experimental conditions. To confirm the requirement of *prrAB* for viability, a second copy of *prrAB* was introduced into the *M. tuberculosis* H37Rv chromosome, generating *M. tuberculosis* STS16. After a single *M. tuberculosis* STS16 mycobacteriophage transduction with the phSH270 Δ*prrAB*::*hyg* thermosensitive mycobacteriophage, 2 of 18 clones (designated STS22 and STS23), or 11%, harbored a mutated wild-type *prrAB* locus (Fig. 3). Thus, the presence of an ectopic *prrAB* locus permitted the disruption of the chromosomal *prrAB* genes and confirmed the essentiality of this regulatory system.

Comparative genomics of the *prrAB* operon in H37Rv, Mt103, and Mt21D3. Given our results that the *prrAB* system is essential in H37Rv and that a *prrA* transposon mutant in the *M. tuberculosis* Mt103 clinical strain has been described (7), we wanted to investigate if different *M. tuberculosis* strains had differential requirements for the PrrAB regulatory system. In order to verify the Mt103 and Mt21D3 strains and to compare the sequence with *M. tuberculosis* H37Rv, we amplified and sequenced the intergenic regions upstream of *prrA* and the *prrA* and *prrB* ORFs in *M. tuberculosis* Mt21D3 and Mt103. Our sequencing results indicated that Tn*5367* inserted 14 bp upstream of the *prrA* start codon in Mt21D3, which is slightly further upstream than previously reported (5 bp upstream of the *prrA* ORF) (7). Compared to H37Rv, there were three nucleotide differences that existed in the Mt103 *prrA* and *prrB* genes—*prrA* C_{221} to T_{221}, *prrB* C_{281} to T_{281}, and *prrB* C_{848} to T_{848}—leading to three amino acid changes, PrrA T_{74} to M_{74}, PrrB S_{94} to F_{94}, and PrrB T_{283} to I_{283}, respectively. No frame shifts or deletions were detected when the Mt103 *prrA* and *prrB* sequences were compared with the H37Rv *prrA* and *prrB* sequences, revealing 711-bp and 1,341-bp ORFs, respectively, in both strains (Fig. 1E). Southern blot analyses, using *prrA* and *aph* (Km) probes, revealed hybridization patterns identical to those in the Ewann et al. (7) study and further confirmed the validity of the Mt103 and Mt21D3 strains (Fig. 4A).

***prrAB* transcription and PrrA protein expression in the wild-type Mt103 and Mt21D3 *prrA* transposon mutant strains.** Due to our failed attempts in generating a Δ*prrAB*::*hyg* knockout mutant, we characterized *prrAB* expression in the *M. tuberculosis* Mt21D3 *prrA* transposon mutant. Although it was previously reported that *prrA* was transiently required for early intracellular multiplication in mouse bone marrow-derived macrophages (7), the authors did not report transcriptional analysis of *prrA* and *prrB* transcripts in the Mt21D3 *prrA* transposon mutant. Based on qRT-PCR analyses, *prrA* and *prrB* transcript levels in Mt21D3 were 30% and 42%, respectively, less than those in wild-type Mt103 (Fig. 4B). These results established that the Tn*5367* insertion upstream of *prrA* in Mt21D3 did not generate a true *prrA*-deficient mutant but rather reduced *prrA* and *prrB* transcription. Moreover, the decrease in *prrB* expression as a result of the transposon insertion is indicative of cotranscription of *prrAB* genes in Mt103 and is similar to the transcriptional results obtained with H37Rv. In accordance with the qRT-PCR results, production of the PrrA protein is also greatly diminished in Mt21D3 compared to that in Mt103 (Fig. 4C). Densitometry analysis revealed that PrrA expression was 71% less in Mt21D3 than in wild-type Mt103 (Fig. 4C). These PrrA expression differences in Mt21D3 are sig-

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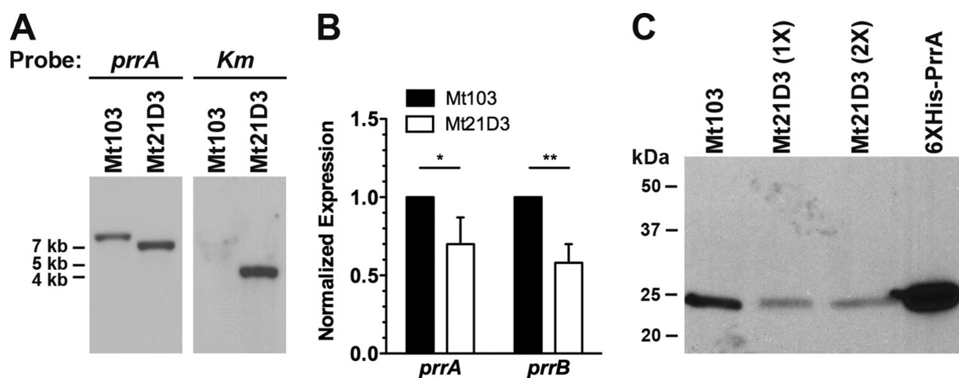

FIG 4 Characterization of *prrA* from *M. tuberculosis* Mt103 wild-type and Mt21D3 *prrA* transposon mutant strains. (A) Genomic DNA from Mt103 and Mt21D3 was digested with BamHI, which is present within Tn*5367* (7), and hybridized with the *prrA* gene or the Km resistance (*aph*) cassette. Based on BamHI sites located within Tn*5367* and flanking the *prrA* and *prrB* genes (7), the expected sizes of the hybridized DNA fragments are as follows: *prrA* (Mt103), 7,801 bp; *prrA* with Tn*5367* inserted in the promoter (Mt21D3), 6,607 bp; and *aph* (Mt21D3), 4,292 bp. Molecular size markers (in kilobases) are shown on the left. (B) qRT-PCR analysis of *prrA* and *prrB* expression in Mt103 and Mt21D3. Results were normalized with respect to 16S rRNA expression and are shown as expression differences from results for the Mt103 control sample, which were assigned a value of 1. The data represent the means ± SD of data from three independent experiments. *, *prrA* P value = 0.0378; **, *prrB* P value = 0.0037. (C) Western blot analysis of whole-cell lysates from *M. tuberculosis* Mt103 (1×, 25 μg) and Mt21D3 (1×, 25 μg; 2×, 50 μg) strains probed with polyclonal anti-PrrA antibody, with the purified His-tagged PrrA protein (40 ng) serving as a control.

nificant considering that PrrA levels in H37Rv were relatively stable regardless of the growth phase or hypoxic conditions (Fig. 2C). Further investigations into defining the PrrAB regulon and determining the role of the PrrAB system in *M. tuberculosis* stress adaptation will be critical in assessing the basis of *prrAB* essentiality.

DISCUSSION

M. tuberculosis two-component regulatory systems are key facilitators of transcriptional adaptation of mycobacteria in response to dynamic and subtle changes in the environment during culture and during infection. *M. tuberculosis* has 11 genetically linked two-component system genes and eight unpaired (or orphaned) histidine kinase or response regulator genes (15) that orchestrate and coordinate adaptive strategies required for survival. The conservation of the PrrAB regulatory system in all mycobacterial species (28) infers that PrrAB has been evolutionarily selected to play an important role in mycobacterial biology, yet we know nothing about its function. The *M. tuberculosis* PrrB histidine kinase and PrrA response regulator represent a functional, cognate two-component signal transduction circuit (22). Autophosphorylation of the PrrB histidine kinase domain was readily demonstrated, and subsequent transphosphorylation of PrrB was rapid and highly specific (8, 22). Here we describe transcriptional analysis of the *prrAB* system and, most important, provide evidence that the PrrAB system is essential for mycobacterial viability. We should note that an identifiable transposon site hybridization (TraSH) signal in the *prrB* gene suggests that the histidine kinase gene can sustain a mutation (25); however, attempts to independently delete the *prrB* gene have not yet been pursued. The induction of *prrAB* under conditions of nitrogen limitation potentially links its function to regulation of nitrogen metabolism.

With this study, we have determined that *M. tuberculosis* harbors not only one but two response regulators, MtrA and PrrA, that are essential for viability. The previously determined essential *mtrA* response regulator gene is transcribed during *M. tuberculosis* growth in macrophages (15, 31), and its overexpression prevents *M. tuberculosis* multiplication in macrophages and mouse lungs and spleens (9). Interestingly, crystal structures of the PrrA and MtrA response regulators show remarkable similarities (10, 22). Both PrrA and MtrA have N-terminal phosphorylation receiver domains with a classic α/β fold and a C-terminal effector domain with a winged helix DNA-binding motif (10, 22). Of all crystallized response regulators described to date, the domain orientation of MtrA is most similar to that of PrrA, with respective inactive domain orientations providing a barrier to activation (3, 10).

Extensive structural investigations have classified response regulators into two categories: (i) readily phosphorylatable (e.g., *Thermotoga maritima* DrrD and *E. coli* PhoB) and (ii) poorly phosphorylatable (e.g., *M. tuberculosis* PrrA and MtrA) (3). Response regulators that are poorly phosphorylated exhibit significant interfaces that exist between the N-terminal phosphorylation receiver domain and the C-terminal DNA-binding domain, thus occluding phosphorylation, stabilizing the inactive conformation of the receiver domain, and sterically inhibiting the helix domain from binding DNA (3, 11). While these substantial interdomain interactions prohibit phosphorylation via small molecule phospho donors (e.g., acetyl phosphate), PrrA domain interactions do not inhibit phosphorylation via its cognate PrrB histidine kinase (3). Structural evidence indicates that unphosphorylated PrrA will not bind DNA (3, 11). However, *in vitro* studies have demonstrated that unphosphorylated, recombinant PrrA binds the *prrAB* promoter region and autoregulates expression (8). Additionally, in support of the reported structural properties, PrrA phosphorylation enhanced DNA binding (8). Taken altogether, these results reveal dynamic, inherent regulatory control of PrrA response regulator function and that maintenance of environmental signal specificity is crucial for PrrAB activation and DNA binding.

Previously, Ewann et al. (7) reported that a *prrA* transposon mutant of the *M. tuberculosis* Mt103 clinical strain, designated Mt21D3, harbors a Tn*5367* transposon inserted 5 bp upstream of the predicted *prrA* start codon. While intracellular growth of Mt21D3 in mouse bone marrow-derived macrophages was impaired during the first 6 days of infection, with 10-fold decreases in CFU compared to levels of the wild-type strain, Mt103, growth recovery of the Mt21D3 mutant was similar to that of Mt103 at day 9 (7). Moreover, transcription of a *prrA-gfp* transcriptional fusion in *Mycobacterium bovis* BCG-infected mouse bone marrow-derived macrophages revealed a 2-fold increase in fluorescence intensity over background levels during the first 3 days of infection (7). After 9 days of intracellular multiplication, *prrA-gfp* expression returned to background levels, indicating transient expression of *prrA* during early phases of intracellular growth (7). These data correlate with our previous reports demonstrating that *prrA* is expressed after 48 h of *M. tuberculosis* growth in human peripheral blood monocyte-derived macrophages (12, 15).

In light of our results revealing the essentiality of the PrrAB two-component system in *M. tuberculosis* H37Rv, it was imperative to evaluate its requirement in other *M. tuberculosis* strains. Our results from sequence comparisons between the wild-type *M. tuberculosis* H37Rv and Mt103 strains revealed one and two nucleotide differences in *prrA* and *prrB*, respectively. We also determined that the Tn*5367* transposon inserted 14 bp upstream of *prrA* in Mt21D3, rather than 5 bp as described previously (7). Furthermore, the Tn*5367* insertion did not knock out *prrA* expression completely in *M. tuberculosis* Mt21D3 but rather decreased *prrA* and *prrB* expression. Accordingly, rather than the absence of the PrrA protein in the Mt21D3 mutant, we observed the presence of the PrrA protein, albeit at levels that were considerably diminished compared to those in the wild-type Mt103 strain. Since *prrA* is expressed during *M. tuberculosis* intracellular growth (12, 15), diminished *prrA* and *prrB* levels in Mt21D3 likely led to the detected intracellular growth deficiencies (7). While it is possible that PrrA is transiently required during early infection events, we cannot exclude the possibility that the observed loss of phenotype at later time points in Mt21D3 occurred as a result of residual PrrA activity in the mutant. Based on our determinations that the *prrAB* two-component system is essential for viability, the Mt21D3 strain could serve as a suitable *prrA* knockdown expression strain for future studies. Given the potential role of *prrA* in *M. tuberculosis* intracellular adaptation, its expression during intracellular growth in human macrophages (12, 15), and its requirement for early intracellular multiplication in murine macrophages (7), further investigations into the basis of *prrAB* essentiality are warranted.

The determination that *prrA* and *prrB* represent a polycistronic operon is not unexpected, since genetically linked two-component system genes are commonly cotranscribed. Comparative analysis of *prrA* and *prrB* transcripts at different growth phases showed maximal levels during exponential growth, with

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greatly diminished expression in stationary phase. The discordance of *prrA* transcript levels with protein levels in actively replicating exponential-phase cultures versus slow-growing stationary phase brings forth the possibility that the PrrA protein is stable and involved in transcriptional regulation during stationary phase. Although much work needs to be done to validate this hypothesis, significant induction of *prrAB* transcripts and PrrA protein levels during nitrogen limitation provides valuable insights into PrrA function. Nitrogen-limiting conditions are typically induced either by limiting the nitrogen source in the culture medium or by blocking assimilation/utilization of the nitrogen source. In a previous report, Amon et al. (2) demonstrated that exogenous addition of MSX to *M. smegmatis* cultures in Middlebrook 7H9 medium induced a cellular nitrogen starvation response at the transcriptional level. MSX inactivates glutamine synthetase (GS) and consequently blocks nitrogen metabolism and impairs ammonia assimilation (2, 18, 21). Interestingly, another key function associated with GS is the synthesis of poly-L-glutamate/glutamine, a virulence-associated *M. tuberculosis* cell wall component. Inhibition of GS via MSX showed correlation with the decreased synthesis of poly-L-glutamate/glutamine in the *M. tuberculosis* cell wall (13). Using the model for nitrogen limitation described by Amon et al. (2), we observed that MSX treatment resulted in a dramatic increase in the transcription of *prrA* and *prrB*, suggesting that this two-component system regulates genes that respond to nitrogen availability or modulates acclimation to nitrogen limitation. Considering the essentiality of the PrrAB system for *M. tuberculosis* viability, the link between PrrAB regulation, GS activity, and cell wall structure prompts further investigation. It should be noted that MSX-treated cultures analyzed in this study maintained glucose as a carbon source. It is known that nitrogen-starved cells that have access to a carbon source (in this case, glucose) exhibit substantial metabolic activity compared to cells subjected to carbon and nitrogen starvation or carbon starvation alone (1); hence, it is plausible that PrrA may also be involved in carbon-derived metabolism.

The global response to nitrogen starvation has been previously studied in *M. tuberculosis* as a model mimicking persistence (5). In this 6-week nitrogen deprivation model, wherein cells exhibit little or no replication, a low respiration rate, and no loss of viability, the stringent response is induced while aerobic respiration, translation, cell division, and lipid biosynthesis are decreased (5). Although *prrA* was not identified in the Betts et al. (5) study, the method of mediating a nitrogen starvation environment was different from the nitrogen-limiting conditions that we used in this study. Given the complex nitrogen deprivation response and its potential link to persistence mechanisms, it will be pertinent to investigate the underlying function of the essential PrrA response regulator during nitrogen-limiting conditions and its influence on *M. tuberculosis* viability, pathogenesis, and persistence.

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