M. tuberculosis AlkX Encoded by rv3249c Regulates a Conserved Alkane Hydroxylase System That Is Important for Replication in Macrophages and Biofilm Formation

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ABSTRACT  Mycobacterium tuberculosis is a highly specialized human pathogen. The success of M. tuberculosis is due to its ability to replicate within host macrophages, resist host immune responses, and ultimately enter a persistent state during a latent tuberculosis infection. Understanding how M. tuberculosis adapts to and replicates in the intracellular environment of the host is crucial for the development of novel, targeted therapeutics. We report the characterization of an M. tuberculosis mutant lacking Rv3249c, a TetR transcriptional regulator. We show that Rv3249c directly represses the adjacent alkB-rubA-rubB operon encoding an alkane hydroxylase/rubredoxin system. For consistency with related systems, we have named the rv3249c gene alkX. The alkX mutant survived better than wild-type M. tuberculosis inside macrophages. This could be phenocopied by overexpression of the alkB-rubAB locus. We hypothesized that the improved intracellular survival phenotype is a result of increased fitness of the mutant; however, we found that the alkX mutant had a defect when grown on some host-associated carbon sources in vitro. We also found that the alkX mutant had a defect in biofilm formation, also linked to the overexpression of the alkB-rubAB genes. Combined, these results define the primary role of AlkX as a transcriptional repressor of the alkB-rubAB operon and suggest the operon contributes to intracellular survival of the pathogen.

IMPORTANCE  Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is the leading cause of death worldwide due to a single infectious agent. It is important to understand how M. tuberculosis adapts to and replicates in the intracellular environment of the host. In this study, we characterized the TetR transcriptional regulator Rv3249c and show that it regulates a highly conserved alkane hydroxylase/rubredoxin system. Our data demonstrate that the AlkBRubAB system contributes to the success of the bacterium in host macrophages.

KEYWORDS  Mycobacterium tuberculosis, transcription factor, alkane hydroxylase, rubredoxin, biofilm

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is the leading cause of death worldwide due to a single infectious agent. The success of M. tuberculosis as a human pathogen can be attributed to its ability to replicate in macrophages, evade host immune responses, and establish a latent infection. Understanding the mechanisms and regulatory processes that underly the ability of M. tuberculosis to survive in the host and establish dormancy is key to the development of novel therapeutics that target latent TB infections.

M. tuberculosis metabolism during infection has been intensely researched. Genetic studies characterizing M. tuberculosis mutants revealed that M. tuberculosis utilizes both host fatty acids and cholesterol as carbon sources when growing intracellularly. Coupling of fatty acid β-oxidation with the glyoxylate cycle is required for successful utilization of fatty acids, as demonstrated by the essentiality of isocitrate lyase 1 and 2 (1). Similarly, the Mce4 cholesterol import system is required for M. tuberculosis virulence (2). Recent work showed that the Mce1

Editor Petros C. Karakousis, Johns Hopkins University School of Medicine
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The authors declare no conflict of interest.
Received 26 May 2022
Accepted 18 July 2022
Published 8 August 2022
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transporter imports fatty acids and that there is interplay between the utilization of these two carbon sources (3). Earlier metabolomic studies also highlight the ability of M. tuberculosis to utilize multiple carbon sources at once (4).

We report the characterization of an M. tuberculosis mutant lacking Rv3249c, a member of the TetR family of transcriptional regulators. We show that Rv3249c represses the operon encoding the alkane hydroxyylase AlkB and the rubredoxins RubA and RubB. AlkB is a predicted alkane hydroxylase belonging to a family of widely distributed integral membrane non-heme diiron mono-oxygenases that permit the utilization of medium- and long-chain (C5–C16) alkanes as a carbon source. AlkB proteins also have homology with membrane-bound fatty acid desaturases (5). M. tuberculosis AlkB has 40% and 44% identity to the characterized alkane hydroxylases from Pseudomonas putida and Pseudomonas aeruginosa, respectively. Using a heterologous expression system, Smits et al. showed that M. tuberculosis AlkB permitted growth of P. putida and P. fluorescens AlkB mutants on C10-12 and C12-16 alkane vapors, demonstrating conserved alkane hydroxylase function (6). However, M. tuberculosis was not able to utilize alkanes in vitro, revealing potential differences between the species (7).

In addition, AlkB proteins typically require one rubredoxin and a rubredoxin reductase. Rubredoxins are iron-sulfur cluster-containing redox-active proteins that shuttle electrons from alkane hydroxylases. M. tuberculosis RubA and RubB were identified as AlkG1- and AlkG2-type rubredoxins, respectively, based on their ability to complement growth on n-octane vapor as a carbon source in a P. putida GPO1 rubredoxin Rd2 mutant (8). Recent in vitro biochemical studies showed that RubB can function as a redox partner for several cytochrome P450 proteins, suggesting a role in cholesterol or fatty acid metabolism (9). The M. tuberculosis gene encoding a rubredoxin reductase has not been identified, and there are no likely candidates in the immediate vicinity of alkB-rubAB.

The gene encoding the Rv3249c transcriptional regulator is immediately downstream of the alkB-rubAB genes in M. tuberculosis. Since this organization is similar to that in other organisms where the TetR regulator homologue is known as AlkX (10), we named rv3249c alkX. We report here that M. tuberculosis AlkX represses alkB-rubAB expression. We show that the alkX mutant survives better than wild-type M. tuberculosis in macrophages, which can be phenocopied by the overexpression of AlkBRubAB. We show that the alkX mutant has impaired biofilm formation, also due to overexpression of AlkBRubAB. These studies define the primary role of AlkX in regulating the AlkBRubAB system.

RESULTS

_rv3249c encodes the transcription factor AlkX, which controls the expression of the alkB-rubAB genes._ AlkX was identified as a potential regulator of the lipid transporter MmpL proteins in published chromatin immunoprecipitation sequencing (ChIP-seq) studies (11, 12). Using electrophoretic mobility shift assay (EMSA), we previously showed that AlkX bound to inter- and intragenic regions of the _mmpL3_ and _mmpL11_ genes (13). To further characterize AlkX, we generated a hygromycin-marked deletion mutant in the M. tuberculosis H37Rv background. The mutation of _alkX_ was verified by PCR amplification of the genomic locus followed by sequence analysis (Fig. 1A).

To examine the function of AlkX as a transcriptional regulator of the _mmpL3/mmpL11_ locus, we quantified expression of _mmpL3_ and _mmpL11_ in wild-type and alkX mutant strains using reverse transcription-quantitative PCR (qRT-PCR). There was no difference in expression of either gene between the wild-type and mutant strains (Fig. 1B). This result likely reflects complex regulation of these genes since we showed that a number of transcription factors bind the promoter or intragenic regions of the _mmpL3/mmpL11_ locus, including Rv1816, Rv0302, Rv1049, and Rv0687 (13, 14).

To identify the AlkX regulon, we performed RNA-seq of wild-type and alkX mutant M. tuberculosis. Using this analysis, we found 41 genes upregulated in the alkX mutant compared to wild-type M. tuberculosis (Table 1). Of the upregulated genes, seven encode other transcription factors. ChIP-Seq data indicate that one of these, the transcriptional regulator Rv1990c, is likely directly regulated by AlkX (15). There were 4 genes downregulated in the alkX mutant compared to wild-type M. tuberculosis (rv3738c and rv3740c to 3742c). The most highly upregulated gene was _whiB7_; however, we did not observe significant differences between wild-type
and alkX mutant M. tuberculosis when we tried to confirm these results using qRT-PCR, and there is no ChIP-Seq evidence of direct binding to this genomic locus. Interestingly, the genes directly upstream of rv3249c comprising alkB, rubA, and rubB were upregulated 7 to 9-fold. Reexamination of the AlkX ChIP-Seq data revealed a significant peak at chromosomal locus 3632057, which corresponds to the DNA region upstream of alkB. Combined, these data suggested that AlkX directly regulates the upstream adjacent genes. We performed qRT-PCR to quantify expression differences in rubB and alkB between the wild-type and alkX mutant strains. Both genes were significantly upregulated in the alkX mutant compared to wild-type M. tuberculosis (Fig. 1C). Wild-type expression levels of rubB and alkB were restored by complementation of the alkX mutant via the integrative plasmid pMV306alkX. These data suggest that AlkX is a repressor of the adjacent genetic locus consisting of rv3250 to 3252c.

The rv3250 to rv3252c genes encode the AlkB alkane-monoxygenase and rubredoxins A and B, and the adjacent rv3253 gene encodes a putative cationic amino acid transporter. The alkB-rubAB genes have overlapping start and stop codons, suggesting that they are transcribed as an operon. There are only 108 bases between the stop codon of the rv3253c gene and the start codon of alkB. To determine if alkB-rubAB is cotranscribed, and assess whether rv3253c is also part of the operon, we performed reverse transcriptase PCR (RT-PCR). Our analysis confirmed that alkB-rubAB comprises an operon but that rv3253c is not cotranscribed (Fig. 2A, reactions 2 and 3). Despite the overlapping stop codon of rubB and alkX, we did not observe a product with RT-PCR for the rubA-alkX (reaction 1). This result may be due to small amounts of the transcript or an additional start site for alkX expression. Indeed, in addition to the transcription start site (TSS) upstream of alkB, we mapped a TSS for alkX within the rubA gene using 5’ rapid amplification of cDNA ends (RACE). This TSS is 205 nucleotides (nt) upstream of the alkX start codon and is included in our complementation vector.
To investigate binding of AlkX to the promoter of the AlkB-rubredoxin locus, we performed EMSA analysis. We observed a concentration-dependent shift of the rv3252c (alkB) promoter probe, but not the rv3253c promoter probe (Fig. 2B). As a control, we added increasing amounts of cold probe to the alkB EMSA. We observed release of the Dig-labeled probe consistent with specific binding of AlkX to the alkB probe. Combined, our results demonstrate that alkB-rubAB are cotranscribed and that AlkX directly controls the expression of the adjacent alkB-rubAB genes.

The alkX mutant survives better than wild-type M. tuberculosis in macrophages. We investigated the fitness of the alkX mutant in the intracellular environment of the macrophage. We found that the alkX mutant survived better than wild-type M. tuberculosis in murine bone marrow-derived macrophages, and this phenotype was more pronounced in interferon-gamma (IFN-γ) activated macrophages (Fig. 3A). Complementation restored wild-type survival. This result suggests that overexpression of the AlkBRubAB system in the intracellular environment is advantageous to the bacterium. To directly test this possibility, we performed macrophage infections with wild-type M. tuberculosis (H37Rv/pVV16) and a strain that overexpresses alkB-rubAB from the strong, constitutive hsp60 promoter of pVV16 (H37Rv/pVV16 rv3250-rv3252c). The overexpression strain survived and replicated better than wild-type M. tuberculosis in resting and activated macrophages (Fig. 3B). Combined, these results indicate that the

| Gene name | Log2 fold change | Protein | Adjusted P value |
|-----------|-----------------|---------|------------------|
| whiB7     | 6.56396615      | Transcriptional regulator WhiB7 | 1.23E-27 |
| rv3196A   | 3.76239378      | Hypothetical protein | 1.77E-30 |
| erm(37)   | 3.71324331      | 23S rRNA [adenine(2058)-N(6)]-methyltransferase | 7.06E-33 |
| rubB      | 3.25343629      | Rubredoxin RubB | 3.03E-40 |
| rv1258c   | 2.93972913      | Multidrug-efflux transporter | 7.31E-35 |
| alkB      | 2.89202234      | Transmembrane alkane 1-monooxygenase AlkB | 1.17E-11 |
| eis       | 2.88356158      | Enhanced intracellular survival protein | 2.23E-16 |
| rubA      | 2.84687642      | Rubredoxin RubA | 9.06E-21 |
| mpt70     | 2.75451936      | Major secreted immunogenic protein Mpt70 | 7.37E-28 |
| Rv2876    | 2.66931017      | Transmembrane protein | 5.88E-28 |
| Rv0263c   | 2.66874621      | Hypothetical protein | 5.26E-35 |
| Rv0264c   | 2.51971624      | Hypothetical protein | 1.48E-38 |
| essP      | 2.43283873      | ESAT-6 like protein EssP | 4.93E-06 |
| Rv1265    | 2.41282456      | Hypothetical protein | 3.93E-06 |
| Rv1460    | 2.30803616      | Transcriptional regulator | 9.18E-10 |
| ppsC      | 2.21583437      | Phthiocerol synthesis polyketide synthase type I | 5.32E-09 |
| Rv2034    | 2.18714561      | ArsR family HTH-type transcriptional repressor | 0.001 |
| ppsB      | 2.17492875      | Phthiocerol synthesis polyketide synthase type I | 1.03E-06 |
| Rv0691A   | 2.06239159      | Mycofactocin precursor | 0.006 |
| Rv1815    | 2.01944873      | Hypothetical protein | 0.0001 |
| Rv0887c   | 1.98750328      | Hypothetical protein | 3.85E-09 |
| hfx       | 1.95345472      | GTP-binding protein Hfx | 3.09E-17 |
| Rv2253    | 1.93966121      | Hypothetical protein | 0.011 |
| Rv2348c   | 1.90736052      | Hypothetical protein | 5.54E-08 |
| mpt83     | 1.90467678      | Cell surface lipoprotein | 3.16E-09 |
| Rv2415c   | 1.89390182      | Hypothetical protein | 1.90E-44 |
| Rv2254c   | 1.86717563      | Integral membrane protein | 7.40E-10 |
| rpfC      | 1.83381221      | Resuscitation-promoting factor RpfC | 0.058 |
| Rv0576    | 1.77272136      | Transcriptional regulator | 0.005 |
| cut2      | 1.69741887      | Cutinase | 8.88E-08 |
| Rv2256c   | 1.69077352      | Hypothetical protein | 6.72E-08 |
| rslA      | 1.65906215      | Anti-sigma-L factor RslA | 4.51E-11 |
| sigL      | 1.62999605      | ECF RNA polymerase sigma factor SigL | 0.0001 |
| PPE15     | 1.61607507      | PPE family protein PPE15 | 0.00001 |
| Rv0449c   | 1.58035955      | Hypothetical protein | 2.04E-07 |
| Rv2250c   | 1.57960119      | HTH-type transcriptional regulator | 3.16E-09 |
| prob      | 1.55670483      | Glutamate S-kinase protein | 1.49E-09 |
| dipZ      | 1.54146103      | Integral membrane C-type cytochrome biogenesis protein | 0.008 |
| Rv2828c   | 1.53964006      | Hypothetical protein | 0.012 |
| Rv1816    | 1.52318941      | HTH-type transcriptional regulator | 0.014 |
| Rv1990c   | 1.503676645     | Transcriptional regulator | 1.23E-07 |
AlkBRubAB rubredoxin system regulated by AlkX contributes to M. tuberculosis survival and replication in macrophages. Macrophages, particularly activated macrophages, produce reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) to target intracellular pathogens. Since the rubredoxins A and B were upregulated in the \textit{alkX} mutant, we speculated that the \textit{alkX} mutant may be better equipped than wild-type \textit{M. tuberculosis} to counter ROI and RNI. To test this possibility, we performed in vitro assays exposing the bacterium to H$_2$O$_2$ as a source of ROI and acidiﬁed nitrite as a source of RNI. We did not observe significant differences between the \textit{alkX} mutant and wild-type \textit{M. tuberculosis} (Fig. 3C).

The \textit{alkX} mutant grows slower on fatty acids and glycerol. Since the \textit{alkB-rubAB} genes encode the alkane hydroxylase AlkB and rubredoxins A and B, an alternative explanation for increased survival in the macrophage could be increased growth in the host environment. However, we did not observe a growth phenotype for the \textit{alkX} mutant compared to wild-type \textit{M. tuberculosis} when the mutant was cultured in standard 7H9 ADS (albumin, dextrose, saline) Tween medium (Fig. 4A). \textit{M. tuberculosis} encounters and utilizes multiple host-associated carbon sources during infection, including fatty acids and cholesterol. To
determine if loss of AlkX impacts bacterial growth on these carbon sources, we grew the wild-type, alkX mutant, and complemented M. tuberculosis strains. There was reduced replication on glycerol, acetate, and the long-chain fatty acid palmitate as the sole carbon sources (Fig. 4B, C, and E). On the other hand, the alkX mutant grew like wild-type M. tuberculosis on cholesterol as the sole carbon source (Fig. 4F). The combined results suggest that the alkX mutant has altered carbon metabolism but do not support the model where the increased fitness of the alkX mutant in macrophage infections stems from greater ability to utilize host-derived carbon sources.

The alkX mutant has impaired biofilm formation associated with overexpression of AlkBRubAB. We were initially interested in AlkX as a regulator of the lipid exporter MmpL11, which is required for biofilm formation (13, 16). While we did not observe an effect
on mmpL3 or mmpL11 expression under planktonic conditions (Fig. 1C), it was possible that their expression was regulated differently under biofilm conditions. We therefore performed qRT-PCR to quantify mmpL gene expression in wild-type, alkX mutant, and complemented M. tuberculosis strains. We did not observe significant differences between the strains (Fig. 5A). To determine if the alkX mutant had altered biofilm formation, we cultured wild-type H37Rv, the alkX mutant, and the complemented strain in Sauton’s medium lacking Tween. The alkX mutant had a visually thinner and weaker biofilm than both the wild type and the complemented strain (Fig. 5B). This corresponded with reduced CV staining of the alkX mutant biofilm material compared to wild-type and complemented strains. Quantification of viable bacteria in each biofilm
culture confirmed that the defect was in pellicle formation rather than growth in Sauton’s medium.

We were curious if the overexpression of alkB-rubAB alone could recapitulate the biofilm phenotype of the alkX mutant. When H37Rv/pVV16 and H37Rv/pW16r3250-52c were grown as biofilms, the overexpression strain had a defect in biofilm formation similar to that of the alkX mutant (Fig. 5C). This corresponded with reduced CV staining of the overexpression strain biofilm material compared to wild-type strains. Since overexpression of alkB-rubAB was detrimental to biofilm formation, we investigated whether rubB and alkB expression was regulated in biofilm cultures relative to planktonic cultures. Using qRT-PCR, we found that rubB and alkB were downregulated in wild-type M. tuberculosis biofilms relative to planktonically grown bacteria (Fig. 5D). Taken together, our data suggest that overexpression of the AlkB-rubredoxin system impairs biofilm formation.

**FIG 5** The alkX mutant has impaired biofilm formation. (A) qRT-PCR analysis showed no significant difference in the transcription of mmpL3 and mmpL11 between the mutant and wild-type strains when grown as biofilms. (B) Wild-type, alkX mutant, and complemented M. tuberculosis strains were cultured as biofilms and imaged. (C) The wild type and the strain overexpressing the alkB-rubAB operon were cultured in biofilms. In panels B and C, the biofilm material was quantified by CV staining and reported as absorbance at 595 nm. The number of bacteria present in the well were determined by mechanically disrupting the biofilm and plating serial dilutions on 7H10 agar. Student’s t test: ***, P < 0.005. (D) qRT-PCR analysis showed that rubB and alkB transcription is downregulated in biofilm-grown M. tuberculosis relative to planktonic bacteria. The difference between rubB and alkB expression in the biofilm cultures compared with planktonic cultures of M. tuberculosis was significant, Student’s t test: ***, P < 0.005. In all experiments, the averages and standard deviation of three biological replicates are shown.
AlkX Regulates M. tuberculosis alkB-rubAB

DISCUSSION

This work defines the role of AlkX as a repressor of the alkB-rubAB locus. Upregulation of the AlkBRubAB system in the alkX mutant improved M. tuberculosis intracellular survival in resting and activated macrophages. Previous studies also support a role for the alkane hydroxylase system in the host. AlkB mutants were attenuated in a SCID mouse, indicating some role for this pathway during infection (17). Global transcriptomics showed that the alkB-rubAB genes were upregulated in resting and activated macrophages. In vitro stimulation with intracellular signals, such as H2O2 and the free fatty acid palmitic acid, also resulted in alkB-rubA-rubB upregulation (18). Expression of the alkB gene was also upregulated in an in vitro phosphate-buffered saline (PBS) starvation model, suggesting that the alkane hydroxylase system contributes to the bacterium’s adaptation to nutrient restriction (19).

AlkB is a predicted alkane hydroxylase. Alkanes are ubiquitous in nature and are produced by plants, algae, and other organisms in contaminated and noncontaminated environments. As such, the ability to oxidize and degrade alkanes is common in both Gram-positive and Gram-negative organisms (20). Environmental mycobacteria can degrade short alkanes; for instance, Mycobacterium vaccae JOBS hydrolyzed propane and butane (21). While M. tuberculosis possesses AlkBRubAB, the ability of the pathogen to utilize alkanes has not been experimentally demonstrated. M. tuberculosis did not grow on the n-alkane paraffin, whereas M. avium-intracellu lar did, suggesting that this phenotype could distinguish nontuberculous mycobacteria (NTM) from M. tuberculosis in clinical specimens (7). Metabolomic analysis of M. tuberculosis strains identified alkanes and a glycolipid surfactant, D-glycero-L-mannoheptonic acid, that is implicated in facilitating alkane uptake (22). Therefore, the role of alkanes and alkane metabolism in M. tuberculosis remains unclear.

The gene encoding the AlkX transcriptional regulator is immediately downstream of the alkB-rubAB genes in M. tuberculosis. A similar genetic organization also exists for alkB-rubAB genes with a gene encoding a TetR family regulator in Gram-positive Actinobacteria, including Rhodococcus, Nocardia, and Dietzia (23). Recent work from Liang et al. demonstrated that the Dietzia DQ12-45-1bTetR regulator AlkX is a repressor of the locus analogous to M. tuberculosis alkB-rubAB (10). Interestingly, they showed that AlkX DNA-binding activity was reduced in the presence of palmitic acid. They highlighted this result as evidence for a product-positive feedback mechanism since long-chain fatty acids are generated as part of alkane degradation. Supporting this model, we previously showed that M. tuberculosis AlkX cocrystallized with palmitic acid, and addition of palmitic acid to EMSA reduced AlkX DNA-binding activity (13). Combined with our current results, our data support a generalized model where TetR repression of AlkBRubAB in Actinobacteria is subject to product-positive feedback via fatty acids.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The M. tuberculosis wild-type strain H37Rv was obtained from the ATCC. Mycobacterial strains are described in Table 2 and were routinely maintained in Middlebrook 7H9 liquid medium (Difco) with 0.05% Tween 80 or on Middlebrook 7H10 agar (Difco), both supplemented with albumin dextrose salts (ADS) containing 8.1 mg/mL NaCl, 50 mg/mL bovine serum albumin (BSA), and 20 mg/mL dextrose. Glycerol was added to liquid 7H9 to a final concentration of 0.5%. Kanamycin (25 μg/mL) and hygromycin (50 μg/mL) were used for selection when required. For growth on defined carbon sources, strains were grown without shaking in Sauton’s medium (0.5 g/L K2HPO4, 0.5 g/L MgSO4, 4.0 g/L L-asparagine, 0.05 g/L ferric ammonium citrate, and 1.0 mM ZnSO4), with a final pH of 7.0, and 0.05% tyloxapol containing glycerol, fatty acids, or cholesterol (Sigma). The final concentrations used were 4.7% glycerol, 1 mM sodium acetate, 0.5 mM sodium butyrate, 0.05 mM palmitic acid, and 0.05 mM cholesterol. Prior to addition, fatty acids were dissolved to 100 mM in a solution of tyloxapol/ethanol (1:1) at 80°C for 30 min and then added to the medium to a final concentration of 0.05 mM.

The M. tuberculosis rv3249c (alkX) mutant was created via allelic exchange. Upstream and downstream regions of alkX were amplified by PCR using ∆rv3249c 5′/3′ forward/reverse primers (see Table 3 for all primer sequences) and cloned to flank the hygromycin resistance gene in pBSK. The resulting plasmid was linearized and used to transform electrocompetent H37Rv. Transformants were selected on 7H10 agar containing ADS and hygromycin (50 μg/mL). Deletion of alkX was confirmed via PCR using flanking primers followed by sequencing of the resulting PCR product. For complementation, alkX + 568 bp upstream sequence was amplified via PCR with primers and cloned into the integrative vector pMV306kan. The sequence includes the TSS identified in the course of this work, as well as the coding sequence of rubAB. The resulting complementation plasmid was transformed into the M. tuberculosis alkX mutant and transformants selected on 7H10 agar containing ADS, hygromycin (50 μg/mL), and kanamycin (25 μg/mL).
H37Rv/pVV16

25 mM Na citrate, pH 7.0, 0.1 M centrifugation, and the pellet was washed in GTC buffer (4 M guanidine thiocyanate, 0.5% Na-mercaptoethanol) followed by phosphate-buffered saline (PBS)-0.1% Tween 80. Bacteria were disrupted by treatment with lysozyme followed by bead beating in the presence of warm TRizol. RNA was subsequently isolated using DirectZol RNA isolation columns (Zymo). RNA was eluted in 50 µL RNase-free water and then treated with DNase (Ambion) to ensure removal of genomic DNA contamination.

RNA-seq analysis was performed at the Oregon State University Center for Genome Research and Biocomputing core facility. RNA libraries were prepared according to Illumina instructions. RNA was removed using Ribo-Zero rRNA. Stranded RNA library prep was performed on a WaferGen Bio-systems Apollo 324 robot, and libraries were quantified by qPCR. RNA-seq was performed on an Illumina HiSeq 3000 instrument to produce 50-bp single-end reads. Illumina CASAVA v1.8 software used for base calling, and sequence reads were assessed for adapters and quality scores using FastQC. For each sample, sequence reads aligned to the M. tuberculosis H37Rv reference genome (GCF_000195955.2) and quantified using Salmon v1.4.0. Differential expression was assessed using DESeq2 v1.32.0. These data were submitted to the GEO repository, study GSE201641.

qRT-PCR. cDNA was prepared from 500 ng of RNA using the Bioline SensiFAST cDNA synthesis kit according to the manufacturer’s protocol. RNA and quality and concentration were measured on a NanoDrop ND-1000 spectrophotometer. qRT-PCR was performed using a Bioline Sensifast HiRox kit with 100 ng of cDNA according to the manufacturer’s protocol. Samples were run in triplicate, and DNase-treated RNA was used as a negative control. Plates were sealed with ThermalSeal RT optically transparent sealing film (Excel Scientific). qRT-PCR was performed on a 4.0 g/L L-asparagine, 0.05 g/L ferric ammonium citrate, 2.0 g/L citric acid (anhydrous), 4.76% glycerol, and 1.0 mg/L ZnSO₄, with a final pH of 7.0. Biofilms were inoculated to an optical density of 600 nm (OD₆₀₀) of 0.05 in Sauton’s medium and incubated at 37°C/5% CO₂ in tightly sealed polystyrene bottles or 50-mL conical tubes. At 2 weeks, the lids were loosened to permit gas exchange. Biofilms were imaged at 3 weeks postinoculation.

RNA-seq. Mycobacterial RNA was isolated as described previously (24). Briefly, bacteria were harvested by centrifugation, and the pellet was washed in GTC buffer (4 M guanidine thiocyanate, 0.5% Na-lauryl sarcosine, 25 mM Na citrate, pH 7.0, 0.1 M β-mercaptoethanol) followed by phosphate-buffered saline (PBS)-0.1% Tween 80. Bacteria were disrupted by treatment with lysozyme followed by bead beating in the presence of warm TRizol. RNA was subsequently isolated using DirectZol RNA isolation columns (Zymo). RNA was eluted in 50 µL RNase-free water and then treated with DNase (Ambion) to ensure removal of genomic DNA contamination.

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M. tuberculosis biofilms were grown in Sauton’s medium containing 0.5 g/L K₂HPO₄, 0.5g/L MgSO₄, 4.0 g/L l-asparagine, 0.05 g/L ferric ammonium citrate, 2.0 g/L citric acid (anhydrous), 4.76% glycerol, and 1.0 mg/L ZnSO₄, with a final pH of 7.0. Biofilms were inoculated to an optical density of 600 nm (OD₆₀₀) of 0.05 in Sauton’s medium and incubated at 37°C/5% CO₂ in tightly sealed polystyrene bottles or 50-mL conical tubes. At 2 weeks, the lids were loosened to permit gas exchange. Biofilms were imaged at 3 weeks postinoculation.

RNA-seq. Mycobacterial RNA was isolated as described previously (24). Briefly, bacteria were harvested by centrifugation, and the pellet was washed in GTC buffer (4 M guanidine thiocyanate, 0.5% Na-lauryl sarcosine, 25 mM Na citrate, pH 7.0, 0.1 M β-mercaptoethanol) followed by phosphate-buffered saline (PBS)-0.1% Tween 80. Bacteria were disrupted by treatment with lysozyme followed by bead beating in the presence of warm TRizol. RNA was subsequently isolated using DirectZol RNA isolation columns (Zymo). RNA was eluted in 50 µL RNase-free water and then treated with DNase (Ambion) to ensure removal of genomic DNA contamination.

RNA-seq analysis was performed at the Oregon State University Center for Genome Research and Biocomputing core facility. RNA libraries were prepared according to Illumina instructions. RNA was removed using Ribo-Zero rRNA. Stranded RNA library prep was performed on a WaferGen Bio-systems Apollo 324 robot, and libraries were quantified by qPCR. RNA-seq was performed on an Illumina HiSeq 3000 instrument to produce 50-bp single-end reads. Illumina CASAVA v1.8 software used for base calling, and sequence reads were assessed for adapters and quality scores using FastQC. For each sample, sequence reads aligned to the M. tuberculosis H37Rv reference genome (GCF_000195955.2) and quantified using Salmon v1.4.0. Differential expression was assessed using DESeq2 v1.32.0. These data were submitted to the GEO repository, study GSE201641.

qRT-PCR. cDNA was prepared from 500 ng of RNA using the Bioline SensiFAST cDNA synthesis kit according to the manufacturer’s protocol. RNA and quality and concentration were measured on a NanoDrop ND-1000 spectrophotometer. qRT-PCR was performed using a Bioline Sensifast HiRox kit with 100 ng of cDNA according to the manufacturer’s protocol. Samples were run in triplicate, and DNase-treated RNA was used as a negative control. Plates were sealed with ThermalSeal RT optically transparent sealing film (Excel Scientific). qRT-PCR was performed on a

M. tuberculosis biofilms were grown in Sauton’s medium containing 0.5 g/L K₂HPO₄, 0.5g/L MgSO₄, 4.0 g/L l-asparagine, 0.05 g/L ferric ammonium citrate, 2.0 g/L citric acid (anhydrous), 4.76% glycerol, and 1.0 mg/L ZnSO₄, with a final pH of 7.0. Biofilms were inoculated to an optical density of 600 nm (OD₆₀₀) of 0.05 in Sauton’s medium and incubated at 37°C/5% CO₂ in tightly sealed polystyrene bottles or 50-mL conical tubes. At 2 weeks, the lids were loosened to permit gas exchange. Biofilms were imaged at 3 weeks postinoculation.
Bio-Rad CFX96 device, using the comparative threshold cycle (CT) method. The following protocol was used: initial denaturation at 95°C for 1 min and then a 2-step PCR with 40 cycles of 95°C for 5 s and 60°C for 30 s. Gene expression was normalized to that of sigA, and the fold change was calculated using the comparative Ct method (25). Primers for qRT-PCR are listed in Table 3.

Electrophoretic mobility shift assay. Probes were amplified from the H37Rv genome using the primers listed in Table 3. All probes were labeled with digoxigenin using the Roche DIG gel shift kit. For EMSA analysis, 12 nM Dig-labeled probe and the indicated micromolar concentrations of protein were incubated for 45 min at room temperature in the Roche binding buffer modified by the addition of 0.25 mg/mL herring sperm DNA, and 0.75 mg/mL poly(dI-C). Reactions were resolved on a 6% native polyacrylamide gel in Tris-borate-EDTA (TBE) buffer and transferred to nylon membrane. Dig-labeled DNA-protein complexes were detected following the manufacturer’s recommendations. Chemiluminescent signals were acquired using an ImageQuant LAS 4000 system (GE).

Determination of transcriptional start sites. RNA was isolated from planktonic M. tuberculosis H37Rv as described above. Transcriptional start sites of alkX were elucidated using the Invitrogen 5’ RACE system for rapid amplification of cDNA ends v2.0. Briefly, 2 µg of RNAse treated RNA was used to synthesize cDNA using a gene-specific antisense primer (pMV306 comple HindIII), which binds at the 3’ end of alkX (Table 3). Subsequently the RNA was degraded and cDNA was purified over a S.N.A.P column following the kit instructions. Terminal deoxynucleotide transferase (TdT) was used to add a 3’ C-tail to the cDNA, and tailed cDNA was amplified using an abridged anchor primer (provided by the kit) and an antisense gene-specific primer (alkGSP2). The amplified sequence was cloned into a pGEM-T Easy Vector and transformed into DH5α. Sequencing of the cloned insert was performed using the T7 promoter primer.

CV staining of biofilm material. Biofilms were cultured in 50-ml conical tubes. At 3 weeks postincubation, medium was removed using a pipette, and the tubes were washed twice with 5 mL PBS. After washing, 5 mL of 1% crystal violet (CV) was added to each tube and incubated for 15 min. CV staining of biofilm material. Biofilms were cultured in 50-ml conical tubes. At 3 weeks postincubation, medium was removed using a pipette, and the tubes were washed twice with 5 mL PBS. After washing, 5 mL of 1% crystal violet (CV) was added to each tube and incubated for 15 min. CV was removed and washed twice with an equal volume of PBS. Following the washes, 95% ethanol was added to each tube, incubated for 10 min, and removed for analysis. Absorbance of the extracted CV was read at 595 nm with a plate spectrophotometer.

Intracellular survival and bacterial replication in bone marrow-derived macrophages. Bone marrow-derived macrophages (BMMO) were isolated from C57/Bl6 mice and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS) (Gibco), 1.5 g/L sodium pyruvate (Gibco), and 20% L cell-conditioned medium. To activate BMMO, cells were treated with 10 ng/mL IFN-γ overnight. BMMO were infected at a multiplicity of infection (MOI) of 1:1 for 1 h and then washed with medium to remove extracellular bacteria. At the indicated time points, infected macrophages were lysed with 0.1% Tween 80, serially diluted, and plated on 7H10 agar plates to determine the CFU of the surviving M. tuberculosis. IACUC approval for bone marrow macrophage isolation is in place at OHSU.

In vitro stress assays. To determine the sensitivity to H2O2, early log cultures were normalized to 5 × 10^6 CFU ml^-1 and incubated for 4 h in the presence or absence of 5 mM and 10 mM H2O2. Each sample was serially diluted and plated onto 7H10 agar. For sensitivity to RNI, cultures were diluted 1:10 in 7H9 ADS Tween medium, pH 5.5 ± 1.5 mM and 3 mM NaN3, and incubated for 6 days following an established protocol (26). Each sample was serially diluted and plated on 7H10 agar to determine the number of surviving bacteria.

ACKNOWLEDGMENTS

This work was funded by NIH NIAID R01 AI123148 to G.E.P. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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