Isolation of Acid-Inducible Genes of *Mycobacterium tuberculosis* with the Use of Recombinase-Based In Vivo Expression Technology

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A better understanding of mycobacterial gene regulation under certain stress conditions (e.g., low pH) may provide insight into mechanisms of adaptation during infection. To identify mycobacterial promoters induced at low pH, we adapted the recombinase-based in vivo expression technology (RIVET) promoter trap system for use with mycobacteria. Our results show that the TnpR recombinase of transposon γ6 is active in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. We developed a method to perform sequential double selection with mycobacteria by using RIVET, with a kanamycin preselection and a sucrose postselection. A library of *M. tuberculosis* DNA inserted upstream of *tnpR* was created, and using the double selection, we identified two promoters which are upregulated at low pH. The promoter regions drive the expression of a gene encoding a putative lipase, lipF (Rv3487c), as well as a PE-PRGRS gene, Rv0834c, in a pH-dependent manner in both *M. smegmatis* and *M. tuberculosis*. The acid inducibility of lipF and Rv0834c was independent of the stress response sigma factor, SigF, as acid induction of the two genes in an *M. tuberculosis* sigF mutant strain was similar to that in the wild-type strain. No induction of lipF or Rv0834c was observed during infection of J774 murine macrophages, an observation which is in agreement with previous reports on the failure of phagosomes containing *M. tuberculosis* to acidify.

One-third of the world’s population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (15). *M. tuberculosis* survives for prolonged periods within phagosomes of infected macrophages (14). In addition, *M. tuberculosis* can persist within the host in an asymptomatic, latent state and can reactivate years later if the host’s immune system wanes. The exact location of these latent bacilli within the host has been the subject of some speculation and controversy, but a possible site is within the calcified remnants of healed granulomas (21, 28). Considering the magnitude of the threat to public health posed by tuberculosis, research into the pathogenesis of the bacterium is of vital importance. Our present lack of understanding of how *M. tuberculosis* evades host defenses and causes disease remains a limitation in developing improved therapeutic and diagnostic tools against tuberculosis.

Recombinase-based in vivo expression technology (RIVET) is a type of promoter trap first described for use with *Vibrio cholerae* and later with *Staphylococcus aureus* (5, 19). RIVET uses the *Escherichia coli* transposon γ6 recombinase, TnpR, which recombines DNA at specific res sites. DNA sequences between these res sites are lost upon recombination, and antibiotic resistance genes placed between res sites may be used as markers for the recombination event (4).

We adapted this system for use with mycobacteria by placing genes for kanamycin resistance and sucrose sensitivity between the DNA res sites. In the absence of *tnpR* expression, mycobacteria are kanamycin resistant and sucrose sensitive, but after *tnpR* expression from an active promoter, the strain becomes kanamycin sensitive and sucrose resistant. With this system, it is possible to identify differentially regulated promoters of mycobacteria within libraries of promoters cloned upstream of a *tnpR* gene. By preselecting with kanamycin for promoter inactivity, administering a stimulus, and then postselecting for sucrose resistance, promoters activated by the stimulus may be identified. We have used this double selection to search for genes of *M. tuberculosis* which are upregulated under conditions of low pH. Such genes may be important for the pathogenic mycobacteria to resist acid stress during infection, as casing granulomas that form in an infected host may have significantly lowered the pH at their centers (11, 12).

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *Mycobacterium smegmatis* strain mc²6 1·2C, *M. tuberculosis* strain CDC1551, and *E. coli* strain HB288 were the parent strains for all genetic manipulations and transformations. All incubations were carried out at 37°C. The *tnpR* promoter trap plasmid was constructed by inserting a promoterless *tnpR* gene amplified by PCR from plVET6 (4) into the XbaI site of *M. tuberculosis* plasmid pMH94 (18) to create pBS14. *tnpR* was subsequently excised as an XbaI fragment, blunted, and ligated into the PvuII site of pNBV1 (17) to create pBS25. To create a terminator, oligonucleotides containing the sequence for the stem-loop terminator downstream of the *M. tuberculosis* whiB3 gene (Rv3416), an internal *SphI* site, and terminal *SpeI* and *HindIII* sites were annealed and ligated into the *SpeI* and *HindIII* sites of pBS25 to create pBS36 (see Fig. 1).

The indicator plasmid pBS31 was constructed by inserting the sequence for *aph*-res into pMH94. Oligonucleotides BS21 and BS26 containing a DNA res site and terminal *HindIII* and *XmaI* sites were annealed. Oligonucleotides BS25 and BS25 containing a DNA res site and terminal MfeI and *HindIII* sites were annealed. The *aph* gene of pMH94 was PCR amplified by using oligonucleotides containing MfeI and *XmaI* terminal restriction enzyme sites. The product was then ligated to the annealed BS21-BS26 and BS25-BS25 oligonucleotides, cut with *HindIII*, and inserted into the *HindIII* site of pMH94 to create pBS20. The resultant plasmid was cut with MfeI, and a *sacb*-containing fragment from pBS-SK+ was inserted to create pBS31.
FIG. 1. Schematic diagram of the mycobacterial RIVET system used in this study. The elements carried by integrating plasmid pBS31 and episomal tnpR promoter-probe plasmid pBS36 are as follows: res is the recombination site for TnpR resolvase; aph encodes aminoglycoside phosphotransferase, conferring kanamycin resistance (5); gfp::tnpR encodes the TnpR resolvase of transposon y6; oriM is the origin of replication for propagation in mycobacteria; oriE is the origin of replication for propagation in E. coli; hgy confers resistance to hygromycin. RBS, ribosome-binding site for tnpR.

The P<sub>rpsL:tnpR</sub> probe was created by amplifying the acetamide-inducible gene block and promoter from pAGAN11 (23) by PCR and inserting the fragment into the SphI and BspHI sites of pBS14 to create pBS16. The DraI-XbaI fragment of pBS16 containing the P<sub>rpsL:tnpR</sub> fragment was then cloned into EcoRV-XbaI-digested pNBV1, creating pBS18.

The library of CDC1551 genomic DNA cloned upstream of tnpR was created by using pBS36. Total genomic DNA was partially cut with the restriction enzyme NcoI and size fractionated by using a 0.7% agarose gel. DNA with a size of 1.0 kb was extracted and ligated into pBS36 that had been cut with SphI and treated with calf intestinal alkaline phosphatase. Six separate ligations were used to transform E. coli SH288. Two thousand colonies were pooled from each transformation, and the plasmid DNA was isolated by using Wizard Minipreps (Promega). Plasmid DNA from the six individual preparations was then used to transform M. smegmatis mc²6 1·2C containing integrated pBS31.

To analyze promoter regions, we used the gfp promoter-probe plasmid pFPV27 (2). The putative promoter region for lppF from -510 to -626 with respect to the putative start of translation was amplified by PCR. The putative promoter region from the constitutively expressed Rv2415c gene was used as a positive control. The fragments were cut with KpnI and BamHI (sites built into the PCR primers) and ligated into pFPV27 to create P<sub>lppF::gfp</sub>, P<sub>RpsL::gfp</sub>, and P<sub>RpsL:tnpR::gfp</sub>, respectively.

**Genetic selection.** To preselect the library of CDC1551 DNA, recombinant M. smegmatis containing the pBS36 library was grown in 7H9 medium plus kanamycin (25 µg/ml) at pH 7.0 for nine generations. The bacteria were diluted in the same medium, grown to mid-log phase, and pelleted. The bacteria were resuspended in 7H9 medium adjusted to pH 4.5 with HCl and grown for another 3 h. The bacteria were then washed and dished in 7H9 medium at pH 7.0 and plated onto 7H10 agar–hygromycin (50 µg/ml)–10% sucrose to obtain sucrose-resistant colonies. This was repeated for two cultures, at which point 30 clones were analyzed for their acid induction.

**Determination of TnpR activity.** To determine if the promoter trap plasmid pBS36 had background expression, the level of TnpR activity was determined. Both M. smegmatis and M. tuberculosis containing pBS36 and pBS31 or pNBV1 and pBS31 were grown for nine generations in 7H9 medium in the absence of kanamycin selection. The mycobacteria were washed and diluted in 7H9 medium and plated onto 7H10 agar–hygromycin (50 µg/ml)–10% sucrose to obtain sucrose-resistant colonies. All measurements were done in triplicate. To determine if tnpR is inducible in mycobacteria, M. smegmatis bacteria containing pBS18 and pBS31, pBS36 and pBS31, or pNBV1 and pBS31 were grown in 7H9 medium for one generation in the absence of kanamycin selection and in the presence or absence of acetamide. The bacteria were diluted and plated for single colonies onto 7H10 agar–hygromycin and onto 7H10 agar–hygromycin–10% sucrose. The colonies that grew on 7H10 agar–hygromycin were picked onto 7H10 agar–hygromycin–kanamycin to assay for the loss of the aph gene. All measurements were done in triplicate. To determine the inducibility of the P<sub>phoA:tnpR</sub> and P<sub>RpsL:tnpR</sub> promoter fusions, M. smegmatis bacteria bearing these constructs as well as pBS36 were grown to mid-log phase in the presence of kanamycin. The bacteria were pelleted, washed, and resuspended in 7H9 medium at either pH 7.0 or pH 4.5 for 3 h. All bacterial samples were then washed, resuspended in 7H9 medium at pH 7.0, diluted to obtain single colonies, and plated onto 7H10 agar–hygromycin plates. Single colonies were picked onto 7H10 agar–hygromycin–kanamycin plates to assess the loss of kanamycin resistance and hence TnpR production. All measurements were done in triplicate.

**Flow cytometry.** M. smegmatis and M. tuberculosis strains containing pBS36 (P<sub>phoA::gfp</sub>, pFPV27, P<sub>gfp::tnpR</sub>, P<sub>conf::tnpR</sub>, and P<sub>conf:tnpR::gfp</sub>) were grown to mid-log phase at 37°C in 7H9 medium at pH 7.0. The bacteria were then washed and resuspended in 7H9 medium at either pH 7.0 or pH 4.5 and grown at 37°C for 4, 8, and 24 h. Bacteria were then diluted in water and analyzed with a Becton Dickinson FACSCalibur flow cytometry system equipped with an argon laser at a wavelength of 488 nm. The bacteria were detected by side scatter and forward scatter. Fluorescence data were collected by using logarithmic amplifiers.

**Macrophage infection and fluorescence microscopy.** Cell culture was performed by using Dulbecco modified Eagle medium (DMEM; high glucose formula) supplemented with 10% fetal bovine serum, 2 mM glutamine, 15 mM HEPES, and 0.02% NaHCO₃ (all from Life Technologies). All incubations were performed at 37°C and 5% CO₂ in humidified incubators. 3T3 cells were seeded at 10⁵ cells per well in four-well Lab-Tek II chamber slides (Nalg Nunc International) and allowed to settle and adhere for 5 h prior to the addition of gamma interferon (IFN-γ) to some wells, followed by overnight incubation. Some cells were activated by treatment with 400 U of murine recombinant IFN-γ (Invitrogen)/ml for 24 h and 200 ng of lipopolysaccharide (LPS; Sigma)/ml for 2 h prior to infection. Macrophages were infected at a multiplicity of infection (MOI) of 5:1 to 5:10 based on previous experiments. After 24 h, medium was replaced with fresh 7H9 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 15 mM HEPES. Slides were mounted with ProLong Anti-Fade mounting medium (Molecular Probes) and visualized by epifluorescence microscopy on a Nikon E-800 microscope.

**RESULTS**

**RIVET with mycobacteria.** RIVET was adapted for use with mycobacteria. The reporter gene tnpR encodes a recombinase from the E. coli transposon y6 that catalyzes recombination between two DNA res sites, excising the intervening DNA (5). We placed res sites flanking both the aminoglycoside phospho-
transferase (aph) gene, providing kanamycin resistance, and the sacB gene, conferring sucrose sensitivity, on a mycobacterial integrating plasmid called pBS31 (25). pBS31 is a derivative of pMH94 and is able to integrate into the mycobacterial attB locus as a single chromosomal copy (18, 26). tnpR was placed on a mycobacterial shuttle plasmid derived from pNBV1 (17) and electroporated into an M. smegmatis strain containing an integrated pBS31 (Fig. 1).

To test whether tnpR expression can be induced in this mycobacterial system, we placed the promoter and regulatory gene block driving expression of M. smegmatis acetamidase (Pace) upstream of tnpR (23). This promoter together with its upstream regulatory genes, cloned as a 4.2-kb cassette, confers chemical inducibility by the substrate acetamide. M. smegmatis bacteria containing integrated pBS31 and the episomal acetamide-inducible Pace::tnpR fusion were grown in hygromycin–kanamycin–7H9 medium to mid-log phase. The cells were then spun down, washed, and resuspended in either 7H9 medium–hygromycin–0.2% acetamide or 7H9 medium-hygromycin. The cells were allowed to grow for 2 h, at which point they were washed with 7H9 medium and plated onto 7H10 agar-hygromycin plates to obtain single colonies. Single colonies were then patched onto 7H10 agar plates containing kanamycin to determine if, in the absence of antibiotic selection and in the presence of acetamide, kanamycin resistance had been lost. Indeed, as seen in Table 1, acetamide induction of tnpR resulted in a 79% loss of kanamycin resistance as opposed to a 2% loss in the absence of acetamide. We confirmed that this loss was due to the excision of the res-aph-sacB-res sequence by monitoring the sequence via PCR with oligonucleotides that amplify the aph gene. Indeed, more than 95% of those bacteria that were unable to grow on kanamycin had lost the aph gene from their chromosomes (data not shown).

To determine if selection on sucrose was feasible, we repeated the above procedure by inducing the M. smegmatis Pace::tnpR fusion strain with acetamide for 2 h. The cells were washed and diluted to allow the isolation of single colonies and were plated onto 7H10 agar-hygromycin plates with or without 10% sucrose. The 7H10 agar plates should support the growth of all bacteria, while the 7H10 agar-sucrose plates should support the growth of only those bacteria which have lost the sacB gene due to the resolution of the res sites. Indeed, from the uninduced culture, the frequency of sucrose-resistant colonies was 4%. After acetamide induction, the frequency of sucrose-resistant colonies increased to 92%, indicating that the majority of cells had lost the sacB gene in response to acetamide induction (Table 1).

A promoterless expression plasmid, pBS25, containing tnpR was tested for residual expression of tnpR in M. smegmatis. In

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<td>pBS18 (Pace::tnpR)</td>
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* ND, not done.

FIG. 2. Strategy for the identification of acid-inducible promoters of M. tuberculosis by using RIVET-based sequential double selection. The M. tuberculosis chromosomal DNA library cloned upstream of tnpR in pBS36 was first used to transform E. coli, and plasmid DNA was isolated from clones in six pools of 2,000. The plasmid DNA was used to transform M. smegmatis to create six pools. The mycobacteria were preselected by growth in 7H9 medium, pH 4.5, in the presence of kanamycin. The bacteria were then washed and resuspended in 7H9 medium, pH 4.5, containing no kanamycin for 3 h. The mycobacteria were then postselected by growth on 7H10 agar plates containing 10% sucrose. The plasmid DNA was then isolated from M. smegmatis bacteria growing on the 10% sucrose plates. Two rounds of sequential double selection were performed.
the absence of any promoter, this plasmid expressed some \( \text{tnpR} \) such that after nine generations of growth in the absence of any antibiotic selection approximately 20% of the bacteria had lost kanamycin resistance (data not shown). In order to decrease the background expression of \( \text{tnpR} \), we cloned a 54-bp stem-loop transcriptional terminator upstream of the \( \text{SphI} \) cloning site in pBS25 to create pBS36. Background expression of this plasmid after insertion of the terminator was reduced and resulted in only 1% of \( M.\text{smegmatis} \) bacteria having lost kanamycin resistance after nine generations in the absence of antibiotic selection. The background expression was tested in \( M.\text{tuberculosis} \) bacteria as well and was found to be 1% after nine generations of growth in the absence of antibiotics (data not shown).

**Library construction and selection.** The usefulness of \( \text{tnpR} \) as a reporter gene to isolate differentially regulated promoters from a library was assessed by using low pH as a stress condition. We created a library of \( M.\text{tuberculosis} \) DNA cloned upstream of \( \text{tnpR} \) within the mycobacterial shuttle vector pBS36. This library was then used to transform \( M.\text{smegmatis} \) containing an integrated copy of pBS31 and to isolate \( M.\text{tuberculosis} \) promoters upregulated upon exposure to pH 4.5. Restriction analysis of 25 representative \( E.\text{coli} \) clones from the DNA library revealed that 90% of the clones contained \( M.\text{tuberculosis} \) chromosomal DNA inserts. The average size of the insert was approximately 1 kb.

Six pools of 2,000 \( E.\text{coli} \) clones were used to prepare six amplified DNA plasmid libraries. The DNA from the six pools

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**FIG. 3.** (A) Map of the \( M.\text{tuberculosis} \) \( \text{lipF} \) and \( Rv0834c \) genetic regions. The numbers denote the locations of the genes within the \( M.\text{tuberculosis} \) H37Rv genome sequence. Putative promoter regions are shown in black and coding sequences are in gray. Inverted repeat sequences are identified with arrows below the putative promoter regions. Bars below the genetic regions indicate DNA that was cloned upstream of \( \text{tnpR} \) in pBS36 or upstream of \( \text{gfp} \) in pFPV27. A TATAT putative −10 promoter element was identified upstream of \( Rv0834c \). (B) The sequences of the inverted repeats preceding the \( \text{lipF} \) and \( Rv0834c \) genes are shown. Seven inverted repeat sequences similar to the repeat upstream of \( \text{lipF} \) were identified elsewhere in the \( M.\text{tuberculosis} \) genome, and three sequences similar to the \( Rv0834c \) repeat were identified.
was then used to transform *M. smegmatis*, generating six pools of *M. smegmatis* recombinant reporter strains with each pool consisting of approximately 10,000 clones. In order to screen for acid-inducible promoters, we subjected these *M. smegmatis* pools to low-pH stress. We first preselected the library to eliminate promoters which were expressed during vegetative growth at neutral pH. To accomplish this, we isolated clones that grew on hygromycin-kanamycin-7H10 agar plates and

![Flow cytometry traces for *M. smegmatis* transformed with gfp reporter constructs](image)

**FIG. 4.** Flow cytometry traces for *M. smegmatis* transformed with gfp reporter constructs pFPV27 (promoterless gfp), pBEN (P<sub>Rv2415c::gfp</sub>), P<sub>Rv0834c::gfp</sub>, and P<sub>lipF::gfp</sub>. The bacteria were induced by growth in 7H9 medium at pH 7.0 or pH 4.5 for 4 h and analyzed with a Becton Dickinson FACSCalibur flow cytometry system. The x axis represents increasing fluorescence (FL), while the y axis represents the number of events. Filled curves are results for cells at pH 7.0 and open curves are results for cells at pH 4.5.
grew these clones in pools to mid-log phase in the presence of kanamycin and hygromycin in 7H9 medium at neutral pH. Only those clones devoid of promoters or with promoters that are inactive at neutral pH can survive in the presence of kanamycin. The *M. smegmatis* pools were then spun down and resuspended in 7H9 medium at pH 4.5 without kanamycin. After growth for 3 h in acidic medium, the bacteria were washed with 7H9 medium, pH 7.0, and plated onto 7H10 agar containing 10% sucrose and hygromycin. This allowed the growth of any bacteria that had lost the *aph* and *sacB* genes due to an upregulated promoter in response to acid induction.

Bacteria that grew on the sucrose plates were pooled, and plasmid DNA was prepared and used to transform *E. coli* to amplify the plasmid DNA. The amplified plasmid DNA was isolated and used to transform *M. smegmatis* to undergo another round of selection (Fig. 2). After the second round, the bacteria growing on the sucrose plates were pooled and the DNA was recovered and used to transform *E. coli* as before. We focused on 30 clones, each of which was retested for acid induction by the same RIVET assay system by using the single pure plasmid to transform *M. smegmatis* harboring an integrated pBS31. Two clones were found to be reproducibly up-regulated in response to medium at pH 4.5.

These two clones were sequenced and found to contain *M. tuberculosis* DNA corresponding to regions upstream of the genes *lipF* (*Rv3487c*) and *Rv0834c* (Fig. 3A). The levels of resolution induced by exposure to acidic medium were quantitated for the two clones designated *lipF*-tnpR and *Rv0834c*-tnpR. Each clone was tested in triplicate. In the presence of acid, *lipF*-tnpR had a resolution rate of 85%, whereas in the presence of neutral pH it had a resolution frequency of 2%. *Rv0834c*-tnpR had a resolution rate of 55% in the presence of acid and a 22% resolution rate in the presence of neutral pH. As expected, *M. smegmatis* bearing the promoterless pBS36 had no increase in the level of resolution during acid induction.

**Confirmation of acid inducibility by gfp reporter analysis.** In order to confirm the acid inducibility of *lipF* and *Rv0834c*, we used *gfp*, encoding the green fluorescent protein, as a reporter gene (9, 24). We PCR amplified ~500-bp fragments from the 5′ untranslated regions of *lipF* and *Rv0834c* and cloned the products upstream of *gfp* in the promoter-probe plasmid pFPV27 (2) (Fig. 3A). In addition, the putative promoter region of the constitutively active gene *Rv2415c* was placed upstream of *gfp* and a positive control clone called pBEN containing the *Mycobacterium bovis* BCG *hsp60* promoter (P<sub>*hsp60*</sub>) cloned into pFPV27 was also used (27). These plasmids were each used to transform *M. smegmatis*. The bacteria were grown to mid-log phase and acid induced as previously described. The level of acid-induced *gfp* expression in these strains was assayed by the amount of fluorescence produced by using a Becton Dickinson FACScan Calibur flow cytometer. As expected, both *lipF* and *Rv0834c* were induced with 4 h of acid exposure, with *lipF* being more dramatically induced. The induction of *lipF* increased more than 10-fold in response to acid, while the induction of *Rv0834c* increased approximately 2-fold (Fig. 4), in general agreement with the tnpR-derived results. The constitutive controls, P<sub>*hsp60*</sub> and the promoter for *Rv2415c*, were both downregulated in response to acid shock, thus indicating a possible general response to acid shock to reduce transcription of unneeded genes (Fig. 4). The promoterless control reporter plasmid (pFPV27) showed no significant change in expression in response to acid induction. Incubation in medium at pH 4.5 for more than 4 h did not cause an increase in induction of *Rv0834c*; however, the level of *gfp* expression continued to increase slightly up until 22 h for *lipF* (data not shown). *M. smegmatis* containing the P<sub>*lipF*</sub>/*gfp* fusion was visualized under phase-contrast and fluorescence microscopy after exposure to acidic and neutral pH. Fluorescence was detected after exposure to low pH but not after exposure to neutral pH, indicating significant acid induction of the *lipF* promoter (Fig. 5).

**Analysis of promoter regions.** In analyzing the putative promoter regions of *lipF* and *Rv0834c*, we identified two different inverted repeat sequences in the 5′ untranslated regions of the acid-induced genes. An inverted repeat was found within the putative promoter region for *lipF* and consisted of 8-bp half sites (5′-GCACGAAT-3′) separated by a 4-bp spacer (Fig. 3B). A different 9-bp inverted repeat containing one mismatch denoted by lowercase] was identified in the 5′ untranslated region preceding *Rv0834c* (5′-GAAATGGAC-3′) within the *M. tuberculosis* genome, we found several other instances of near-perfect inverted repeat sequences similar to those preceding the *lipF* and *Rv0834c* genes in the 5′ untranslated regions of other genes, as may be seen in Fig. 3B.

**Survival of M. smegmatis under low pH conditions.** To assess the extent of killing during acid induction, *M. smegmatis* was exposed to pH 4.5 for 3, 8, and 22 h. At 3 h there was no appreciable killing of the bacteria (based on comparison of CFU per milliliter in acid-treated and control suspensions of *M. smegmatis*). However, at 8 h approximately 30% of the
bacteria were killed due to the acidic conditions. At 22 h after acid induction, there was a significant level of killing, as 70% of the bacteria were nonviable. Therefore, while acidified medium does not have an immediate effect on the viability of the mycobacteria, over time it becomes bactericidal. Hence, under the selection conditions used in our RIVET assay, there was no appreciable bacterial killing by low pH.

Low-pH inducibility of the lipF and Rv0834c promoters in M. tuberculosis. We assessed the level of acid-induced transcription within M. tuberculosis by using the P_{lipF}::gfp and P_{Rv0834c}::gfp promoter-probe constructs to transform M. tuberculosis. The M. tuberculosis transformants were grown to mid-log phase and acid induced as previously described, and their fluorescence was monitored by using flow cytometry. Again, both promoter regions were induced at pH 4.5. Induction was found to be slower than that in M. smegmatis. While there was induction at 4 h postacidification, the greatest increase in acid induction occurred at 22 h (Fig. 6). Again, P_{lipF} was upregulated approximately 10-fold, while P_{Rv0834c}, being semiconstitutive, was upregulated about 5-fold, an increase similar to that seen in M. smegmatis.

Acid-induced expression of lipF and Rv0834c is not dependent on the M. tuberculosis sigma factor, SigF. To test whether the M. tuberculosis genes lipF and Rv0834c are acid regulated by the stress response sigma factor, SigF, we used the promoter-gfp fusions to transform both an M. tuberculosis strain with a deletion of sigF and its isogenic wild-type parent strain, CDC1551 (6, 13, 20). When the M. tuberculosis transformants were acid induced, there were equivalent levels of induction in the wild-type strains and the sigF mutant strains bearing the P_{lipF}::gfp and P_{Rv0834c}::gfp fusions. These data indicate that SigF does not mediate the acid-inducible expression of these promoters. As expected, there was no difference between the pre- and post-acid induction expression of gfp in either the wild-type strain or the sigF mutant strains bearing the vector control pFPV27 or the P_{hsp60}::gfp plasmid pBEN (data not shown).

lipF and Rv0834c are not induced within the phagosome of J774 murine macrophages. To investigate whether lipF or Rv0834c is induced within the intracellular environment of the macrophage phagosome, M. tuberculosis CDC1551 bacteria harboring pFPV27 (promoterless gfp), pBEN (P_{hsp60}::gfp), P_{Rv0834c}::gfp, and P_{lipF}::gfp. The bacteria were induced by growth in 7H9 medium at pH 7.0 or pH 4.5 for 22 h and analyzed with a Becton Dickinson FACSCalibur flow cytometry system. The x axis represents fluorescence (FL) intensity, while the y axis represents the number of events. Filled curves are results for bacteria at pH 7.0 and open curves are results for bacteria at pH 4.5.
FIG. 7. Expression analysis of lipF and Rv0834c in J774 murine macrophages. Macrophages, activated with IFN-γ and LPS, were infected at an MOI of five bacilli per cell as described in Materials and Methods. After 72 h, cells were fixed, mounted, visualized by epifluorescence microscopy, and compared to bacilli exposed to tissue culture medium alone. Shown are corresponding images of M. tuberculosis Oshkosh strains in activated macrophages (left panels) and in tissue culture medium alone (right panels). Similar levels of fluorescence were observed in the unstimulated macrophages (data not shown).
For this would be “leaky” expression of *tnpR*. We measured the background of our RIVET system to be around 1%. Even this low level of background could generate a large number of false positives. Further modification of the system may reduce the level of background *tnpR* expression.

We were unable to observe the induction of *lipF* or *Rv0834c* during macrophage infection. It is possible that the phagosomal compartment fails to acidify to the degree required for induction. Indeed, one of the tenets of mycobacterial pathobiology has been the prevention of phagosome maturation and subsequent acidification (7, 10, 14, 30, 31). However, it has been suggested that the centers of caseating granulomas can be quite acidic in vivo (11, 12). Therefore, acid-induced genes of *M. tuberculosis* could play a role in survival in such an environment.

In analyzing the putative promoter regions of *lipF* and *Rv0834c*, we identified two unique inverted repeat sequences—one upstream of *lipF* and the other upstream of *Rv0834c*. Inverted repeats similar to that preceding *lipF* were found in seven other 5′ untranslatable regions in the *M. tuberculosis* genome, and repeats resembling that upstream of *Rv0834c* were found preceding three other *M. tuberculosis* genes. Bacterial transcriptional regulators are known to recognize and bind to DNA inverted repeats (1, 22, 29), and this raises the possibility that the repeats in the *lipF* and *Rv0834c* promoter regions may play a role in the transcriptional response to acid stress.

DNA-binding transcriptional regulators may either repress or activate transcription. For example, *Rv0834c* is semiconstitutive and a TATAT sequence can be found 59 bp upstream of the putative start site of translation and 15 bp downstream of the inverted repeat (Fig. 3A). This TATAT sequence is identical to the −10 region of a constitutive promoter identified in a study by Bashyam et al. and could explain the basal level of transcription of *Rv0834c* (3). Binding of a transcriptional regulator in response to acidic stress may then serve to increase the level of transcription beyond the constitutive level. The *lipF* gene is not expressed to any appreciable level in the absence of acidic stress; a transcriptional regulator may serve either to repress transcription in the absence of acidic stress or to activate transcription in the presence of acidic stress. Acid-induced genes such as *lipF* and *Rv0834c* may play a role in the pathogenesis of human tuberculosis because of the association between low pH and the core of granulomatous lesions. Further investigation is warranted to determine whether the inverted repeats play a role in the regulation of *lipF* and *Rv0834c* and to further define the role of these low-pH-responsive genes in infection.

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**REFERENCES**


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