Transcriptional Regulation of Multi-Drug Tolerance and Antibiotic-Induced Responses by the Histone-Like Protein Lsr2 in *M. tuberculosis*

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Multi-drug tolerance is a key phenotypic property that complicates the sterilization of mammals infected with *Mycobacterium tuberculosis*. Previous studies have established that *iniBAC*, an operon that confers multi-drug tolerance to *M. bovis* BCG through an associated pump-like activity, is induced by the antibiotics isoniazid (INH) and ethambutol (EMB). An improved understanding of the functional role of antibiotic-induced genes and the regulation of drug tolerance may be gained by studying the factors that regulate antibiotic-mediated gene expression. An *M. smegmatis* strain containing a lacZ gene fused to the promoter of *M. tuberculosis iniBAC* (*P*iniBAC) was subjected to transposon mutagenesis. Mutants with constitutive expression and increased EMB-mediated induction of *P*iniBAC::lacZ mapped to the *lsr2* gene (MSMEG6065), a small basic protein of unknown function that is highly conserved among mycobacteria. These mutants had a marked change in colony morphology and generated a new polar lipid. Complementation with multi-copy *M. tuberculosis* *lsr2* (Rv3597c) returned *P*iniBAC expression to baseline, reversed the observed morphological and lipid changes, and repressed *P*iniBAC induction by EMB to below that of the control *M. smegmatis* strain. Microarray analysis of an *lsr2* knockout confirmed upregulation of *M. smegmatis iniA* and demonstrated upregulation of genes involved in cell wall and metabolic functions. Fully 121 of 584 genes induced by EMB treatment in wild-type *M. smegmatis* were upregulated (“hyperinduced”) to even higher levels by EMB in the *M. smegmatis* *lsr2* knockout. The most highly upregulated genes and gene clusters had adenine-thymine (AT)–rich 5′-prime untranslated regions. In *M. tuberculosis*, overexpression of *lsr2* repressed INH-mediated induction of all three *iniBAC* genes, as well as another annotated pump, *efpA*. The low molecular weight and basic properties of *Lsr2* (pl 10.69) suggested that it was a histone-like protein, although it did not exhibit sequence homology with other proteins in this class. Consistent with other histone-like proteins, *Lsr2* bound DNA with a preference for circular DNA, forming large oligomers, inhibited DNase I activity, and introduced a modest degree of supercoiling into relaxed plasmids. *Lsr2* also inhibited in vitro transcription and topoisomerase I activity. *Lsr2* represents a novel class of histone-like proteins that inhibit a wide variety of DNA-interacting enzymes. *Lsr2* appears to regulate several important pathways in mycobacteria by preferentially binding to AT-rich sequences, including genes induced by antibiotics and those associated with inducible multi-drug tolerance. An improved understanding of the role of *Lsr2* may provide important insights into the mechanisms of action of antibiotics and the way that mycobacteria adapt to stresses such as antibiotic treatment.

Introduction

*Mycobacterium tuberculosis* appears to generate specific and coordinated transcriptional responses to antibiotic treatment [1,2]. Several broad categories of genes are induced by antibiotics, including a number involved in stress responses and others linked to specific metabolic pathways that are inhibited by antibiotics [1]. The functional roles of antibiotic-induced transcriptional changes are poorly understood. Some changes are likely to be adaptive in that they induce antimicrobial tolerance or are important for intrinsic drug resistance [3–5]. Other changes are likely to be detrimental to the cell and may be ultimately linked to cell death. An improved understanding of the functional role of antibiotic-induced genes may be gained by studying the factors that regulate their expression. Histone-like proteins are reasonable candidates for regulators of antibiotic responses in bacteria because they assist in the control of stationary and exponential phase cell growth and regulate genes that

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Abbreviations: AT, adenine-thymine; EMB, ethambutol; EMSA, electrophoretic mobility shift assay; INH, isoniazid; *P*iniBAC, *iniBAC* promoter; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography

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respond to environmental changes [6,7]. Whether histone-like proteins influence the transcriptional response to antibiotics is not known.

The *M. tuberculosis* iniBAC operon (*iniB* or Rv0341, *iniA* or Rv0342, and *iniC* or Rv0343) encodes an important example of antibiotic-induced genes, including *iniB*, in two mycobacterial species revealed that a small protein called Lsr2 controls *iniBAC* and other antibiotic-induced genes, especially ones related to the cell wall. Lsr2 binds to DNA in a relatively non-specific manner and appears to inhibit certain enzymes that must interact with DNA as part of their function. These properties differentiate Lsr2 from classical regulators of gene expression that bind to specific DNA sequences, and suggest that Lsr2 is a novel histone-like protein. These proteins regulate genes by changing the way DNA is shaped, and, indeed, we found that Lsr2 can change the shape of DNA by introducing a small number of coils into its structure. Our results suggest that Lsr2 is a major regulator of antibiotic-induced responses in mycobacteria.

The *lsr2* Gene Is Required to Repress *PiniBAC* Activity

Previous studies have found an association between antibiotic-mediated induction of the *iniBAC* genes and multidrug tolerance in BCG [3]. We performed transposon mutagenesis studies to identify repressors of *iniBAC* transcription and gain a better understanding of the regulation of drug tolerance. Mutagenesis was performed in NJS20, an *M. smegmatis* Mc^2^155 strain that contained a single copy of the *M. tuberculosis* *PiniBAC* fused to a lacZ reporter inserted into *attP* [8]. NJS20 normally exhibits a subtle light tan-blue color when cultured on media containing X-gal (Figure 1A). We found five strongly blue transposon mutants that had an unusual round and shiny colony morphology (Figure 1A and 1B). This morphology has been previously reported for *lsr2* transposon mutants in *M. smegmatis* (A) Magnification of individual colonies. Left, wild-type NJS20.w (control); center, NJS20.1 (*lsr2* transposon mutant); right, NJS20.1c (complemented *lsr2* transposon mutant).

(B) Overall morphology and color of the two strains. Left, NJS20.w; right, NJS20.1

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Figure 1. Morphology and β-Lactamase Activity of the *lsr2::Tn5370* Mutant in *M. smegmatis*
The activity of the $\text{P}_{\text{iniBAC}}$ in the presence or absence of 5 μg/ml EMB is indicated by $\beta$-galactosidase units.

(A) “Uninduced” cultures without EMB treatment.
(B) “Induced” cultures treated with EMB.

Open columns, $\beta$-galactosidase activity of an NJS20.w control containing a random transposon insertion; dashed columns, the $\text{lsr}2$ transposon mutant strain NJS20.1; closed columns, the complemented mutant NJS20.1c. The mean and standard deviations of at least triplicate experiments of each strain are shown. Note that different scales were used for $\beta$-lactamase units in (A) and (B). doi:10.1371/journal.ppat.0030087.g002

**Figure 2.** Effect of $\text{lsr}2$ Gene Inactivation and Overexpression on Activity of the M. tuberculosis $\text{iniBAC}$ Promoter

We investigated the role of $\text{lsr}2$ in repressing basal and antibiotic-induced $\text{P}_{\text{iniBAC}}$ activity. An NJS20 ($\text{lsr}2$:Tn5370) $\text{lsr}2$ transposon mutant (NJS20.1) was cultured to mid log phase, EMB or control media was added to each culture for 24 h, and lacZ expression was measured. A second NJS20 strain with an intact $\text{lsr}2$ gene containing a random transposon insertion (NJS20.w) was selected from the same transposon library to serve as a control. The results of these experiments showed that levels of $\beta$-galactosidase activity were approximately five times higher in the NJS20.1 $\text{lsr}2$ transposon mutant strain than in the control strain in the absence of antibiotic treatment (Figure 2A). $\text{P}_{\text{iniBAC}}$ was also induced to higher levels in NJS20.1 than in NJS20.w after treatment with EMB (Figure 2B). The NJS20.1 $\text{lsr}2$ transposon mutant was then complemented by overexpressing the M. tuberculosis $\text{lsr}2$ gene (Rv3597c) in pMP167, creating strain NJS20.1c. In the absence of antibiotic treatment, complementation restored the wild-type phenotype and lowered basal lacZ expression to levels not significantly different than those of the wild-type NJS20.w control (Figure 2A). The colony morphology also reverted to normal in the complemented strain (Figure 1A). The complemented strain showed significantly less $\text{P}_{\text{iniBAC}}$ induction than the NJS20.w transposon mutant control in the presence of EMB treatment. In other words, the presence of $\text{lsr}2$ on a multi-copy plasmid actually repressed EMB-mediated $\text{P}_{\text{iniBAC}}$ induction below that of the wild-type strain (Figure 2B). These results indicate that the $\text{lsr}2$ gene controls both basal and antibiotic-induced levels of $\text{iniBAC}$ expression.

**Effect of $\text{lsr}2$ Deletion and Overexpression on Antibiotic Susceptibility**

It has been shown previously that M. smegmatis strains overexpressing $\text{iniA}$ are somewhat more resistant to EMB than controls [3]. We postulated that the NJS20.1 $\text{lsr}2$ mutant would also be more resistant to EMB due to de-repression of $\text{iniA}$. NJS20.1 was indeed more resistant to EMB than either NJS20.w or NJS20.1c using the proportions method of susceptibility testing [10] (Figure 3A). Complementation restored EMB susceptibility to the level of the control strain. We tested all strains with ciprofloxacin in the same manner as the EMB assays to address the possibility that the $\text{lsr}2$ activity is specific to cell wall antibiotics (Figure 3B). All strains were equally susceptible to ciprofloxacin at all concentrations, suggesting that the role of $\text{lsr}2$ is limited to antibiotics that target the cell wall.

**Permeability Studies**

Changes in colony morphology and antibiotic susceptibility can be associated with changes in cell wall permeability. Therefore, it was possible that the observed differences in $\text{P}_{\text{iniBAC}}$ induction could be due to increased entry of EMB into the cell. We measured the cell wall permeability of NJS20.1 to both hydrophilic and hydrophobic compounds by examining permeability to glycerol [carbonyl-C14] and chenodeoxycholic acid [carbonyl-C14], respectively. No change in the intracellular levels of either compound was noted in NJS20.1 compared to control NJS20.w (unpublished data), indicating...
that disruption of lsr2 does not lead to substantial permeability changes.

**Microarray Analysis**

Microarray studies were performed to further investigate the role of lsr2 in transcriptional regulation under baseline and antibiotic-inducing conditions. A new M. smegmatis Δlsr2 strain that contained a complete unmarked deletion of lsr2 (strain NJS22) was created to perform these studies. Three different comparisons were made: Expression of NJS20 was compared to NJS22 expression to identify genes that were up- or downregulated by deletion of lsr2 (comparison 1) (Table S1). NJS20 grown in 7H9 media was compared to NJS20 cultured with EMB to identify genes that were induced by EMB in wild-type M. smegmatis (comparison 2) (Table S2). NJS20 treated with EMB was compared to NJS22 treated with EMB to identify genes that were induced by EMB in a Δlsr2 background and to identify the EMB-induced genes that were further upregulated (“hyperinduced”) by lsr2 deletion (comparison 3) (Table S3). All genes with statistically significant changes (p < 0.05) in gene expression were included in each analysis (Figure 4A). Comparison 1 revealed that 344 genes were upregulated and 286 genes were downregulated by lsr2 deletion. The increased ratio of up- versus downregulated genes was even more remarkable when the analysis was restricted to genes with statistically significant expression changes of 1.5-fold (146 up- versus 103 downregulated) or 2-fold (41 up- versus 19 downregulated) and is consistent with our hypothesis that lsr2 has broad and principally repressive effects on transcription. As predicted by the M. tuberculosis P_MINAC reporter assay, M. smegmatis iniA expression was significantly increased in NJS22 compared to NJS20, although absolute upregulation was only 1.3. Other broad categories of genes that were upregulated in condition 1 included genes involved in cell wall processes, metabolism, and transport (Table S1). Interestingly, stress response genes were not strongly represented among the genes upregulated in this comparison.

The microarray studies allowed us to search for DNA sequences that might represent binding sites for Lsr2 or an associated protein. However, no consensus sequences were identified in alignments of up to 400 bp upstream of the 15 most strongly induced genes. In contrast, these regions were found to be unusually adenine-thymine (AT)-rich (43.2% AT compared to a mean of 32.6% AT in the M. smegmatis genome). Mapping the induced and repressed genes over the entire chromosome revealed a number of chromosomal regions with large clusters of highly upregulated genes (Figure 4B). The upregulated genes within each cluster did not appear to comprise single operons (Figure 4C). As with the 20 most highly induced genes, the regions 400 bp upstream of the upregulated genes shown in Figure 4C were unusually AT-rich (41.4% AT for region 1 and 40.5% AT for region 2). These results are consistent with the hypothesis that lsr2 encodes a protein with relatively non-specific rather than sequence-specific DNA-binding properties that preferentially binds to AT-rich sequences in a manner similar to that of some other histone-like proteins [11].

Our reporter studies had shown that the M. tuberculosis
PiniBAC was upregulated by lsr2 disruption in NJS20.1, induced by EMB in NJS20, and hyperinduced by EMB in NJS20.1. We examined conditions 1–3 to identify the complete complement of M. smegmatis genes that exhibited this expression pattern. As predicted, we found that iniA was significantly upregulated/induced in all three conditions. Interestingly, only ten other genes had similar expression patterns (Figure 4A; Table 2) (p = 0.0001 that this number of genes were not present in all three conditions by chance). This group was overrepresented by genes involved in cell wall biosynthesis, transport, or other cell wall functions, providing a link to iniA, which appears to encode for a pump-associated protein in M. tuberculosis [3].

\[ P_{iniBAC} \text{ was upregulated by } lsr2 \text{ disruption in NJS20.1, induced by EMB in NJS20, and hyperinduced by EMB in NJS20.1. We examined conditions 1–3 to identify the complete complement of } M. \text{ smegmatis genes that exhibited this expression pattern. As predicted, we found that } iniA \text{ was significantly upregulated/induced in all three conditions. Interestingly, only ten other genes had similar expression patterns (Figure 4A; Table 2) (p = 0.0001 that this number of genes were not present in all three conditions by chance). This group was overrepresented by genes involved in cell wall biosynthesis, transport, or other cell wall functions, providing a link to } iniA, \text{ which appears to encode for a pump-associated protein in } M. \text{ tuberculosis [3]. One hundred and twenty-one genes were induced in both condition 2 and 3 (p = 0.0001), indicating that many of the genes that are induced by EMB in wild-type } M. \text{ smegmatis are hyperinduced in a } D\text{lsr2 background. These results support the hypothesis that } lsr2 \text{ is involved in controlling the level of expression of a subset of cell wall–} \]

### Table 1. Plasmids, Strains, Primers and Molecular Beacons Used in This Study

| Category | Name | Relevant Features / Sequences | References |
|----------|------|------------------------------|------------|
| Plasmids | pMV261 | Multi-copy vector encoding kanamycin resistance and containing the hsp60 promoter. | [52] |
|         | pMP167 | Multi-copy vector encoding apramycin resistance and containing the hsp60 promoter. | [53] |
|         | pMV261::lsr2 | pMV261 with M. tuberculosis lsr2 inserted downstream of the hsp60 promoter. | This study |
|         | pMP167::lsr2 | pMP167 with M. tuberculosis lsr2 inserted downstream of the hsp60 promoter. | This study |
|         | pET-30 | pET-30 with M. tuberculosis lsr2 inserted as described in Materials and Methods. | This study |
|         | pET-30:Js2r | pET-30 with M. tuberculosis lsr2 inserted as described in Materials and Methods. | This study |
|         | pCV125 | Integrating vector encoding kanamycin resistance. | [2] |
|         | pGV1898–12 | pCV125 vector containing P<sub>hsp60</sub> fused to lsr2 as described. | [2] |
| Strains | NJS20 | M. smegmatis strain mc<sup>155</sup> containing pGV1898–12 inserted into attP. | [8] |
|         | NJS20.1 | NJS20 with Tn5370 interrupting the lsr2 gene. | This study |
|         | NJS20.1c | NJS20.1 containing pMP167::lsr2. | This study |
|         | NJS22 | M. smegmatis strain mc<sup>155 Δlsr2</sup> (unmarked). | This study |
|         | H37Rv (pMV261) | H37Rv containing pMV261. | This study |
| Cloning primers | pmv261-<i>lsr2</i>F | tttccgagccggtccacatgaga | This study |
|         | pmv261-<i>lsr2</i>R | ctcgctattggacaggtctgag | This study |
|         | pET-30-<i>lsr2</i>F | tttccgagccggtccacatgaga | This study |
|         | pET-30-<i>lsr2</i>R | ctcgctattggacaggtctgag | This study |
|         | F1-<i>lsr2</i>KO | ttcgaacctggcagctgctcttctct | This study |
|         | R13-<i>lsr2</i>KO | tttgacgtcagctgacgcatccttcc | This study |
|         | F2-<i>lsr2</i>KO | ttcggtgcagctgacgcatccttccgtc | This study |
|         | R2-<i>lsr2</i>KO | ttcggtgcagctgacgcatccttccgtc | This study |
|         | F-<i>lsr2</i>SC | Ccaatgtggtggttgcaggtgc | This study |
|         | R-<i>lsr2</i>SC | Ccaatgtggtggttgcaggtgc | This study |
| Molecular beacon assay (forward/reverse, antisense, molecular beacon*) | sigA | ggccagcggcgcgaccctgtg / gtccaggtagtggccaggacc | [59Rsqb; |
|         | 165 | gctgctacggggtggtggtg / gccgcgtaccgtcgtgcgtgt | This study |
|         | iniA | caattggcggtgtctctaggg / gaagcaagttgtcctgctgct | This study |
|         | iniB | egcgcgaacacacacacac / ctgcgcgcacacacacac | This study |
|         | iniC | cggccgccacccacacacac / cggccgcacccacacacac | This study |
|         | Lsr2 | ttcgctctacagtctcttctg / gcagacgcgcgcgcgcgctgct | This study |
|         | efuA | ggtgtgctctacagtctcttctg / gcagacgcgcgcgcgcgctgct | This study |

*Molecular beacon arm sequences indicated by capital letters.

**FAM**, 6-carboxyfluorescein.

[^4](4-dimethylaminophenylazo)benzoic acid] succimidyl ester (DABCYL).

*Tertafluoresceine.

doi:10.1371/journal.ppat.0030087.t001
active antibiotic-induced genes. Interestingly, only 21 genes were upregulated by condition 1 and induced in condition 2 ($p = 0.81$), while 41 were upregulated by condition 1 and induced in condition 3 ($p = 0.0001$). These results indicate that many of the genes controlled by lr2 are not related to EMB treatment, suggesting that lr2 is involved in the control of a broad range of cellular processes.

Overexpression of lr2 Downregulates the iniBAC Operon and the efpA Gene in M. tuberculosis

The observation that lr2 participates in repression of the M. tuberculosis PiniBAC in M. smegmatis suggested that lr2 might perform a similar function in M. tuberculosis. We were unable to generate an M. tuberculosis Δlr2 strain using allelic exchange methods to study this question directly [12]. Although this result cannot be taken as proof for gene essentiality, it is consistent with Himar1-based transposon mutagenesis studies, which indicate that lr2 is essential in M. tuberculosis H37Rv [13], and with the results of another transposon mutant screen in M. tuberculosis [14] in which Lsr2 insertions were only detected at the extreme 3-prime end of the gene (R. McAdam, personal communication). We then decided to study the effect of lr2 overexpression in M. tuberculosis in strain H37Rv by overexpressing M. tuberculosis lr2 using the multi-copy plasmid pMV261::lr2 (creating strain NJT18). NJT18 overexpressed lr2 approximately 70-fold, as confirmed by quantitative PCR (unpublished data).

We cultured NJT18 and a wild-type H37Rv control strain to mid log phase, incubated the cultures with INH at a final concentration of 1.0 ug/ml, and measured expression of inib, iniA, and iniC, by quantitative PCR. We found that overexpression of lr2 downregulated INH-mediated induction of the iniBAC genes in NJT18 compared to the H37Rv (pMV261) control (Figure 5). These results are consistent with our discovery that overexpression of lr2 in M. smegmatis repressed EMB-mediated induction of PiniBAC and they confirm the role of lr2 in repressing gene expression in M. tuberculosis.

We examined the effect of lr2 overexpression on kasa, efpA, and inha expression in order to determine whether lr2 acted specifically on the iniBAC operon or whether it had a more global effect (Figure 5). The kasa and efpA genes were examined because both genes are induced by INH. Furthermore, efpA has been annotated as a efflux pump [15], suggesting that it might have functions analogous to the iniA-associated pump. Expression of inha was studied as a control because INH does not induce this gene. We found that inha expression was not affected by lr2 overexpression. This indicates that lr2 overexpression does not cause generalized repression of all gene expression in M. tuberculosis. INH-mediated induction of efpA and kasa were modestly downregulated in the lr2 overexpression strain, although the downregulation of kasa induction did not appear to be statistically significant.

Inactivation of lr2 Affects Lipid Composition

Inactivation of lr2 resulted in a remarkable change in colony morphology in M. smegmatis (Figure 1). A similar observation has been made previously by Chen et al. [9]. Colony morphology is often associated with a change in the cell wall structure [16–18]. Chen et al. noted that disruption of lr2 in M. smegmatis was associated with the disappearance of two apolar lipids. We analyzed the lipid composition of the NJS20.1 lr2 mutant in this study compared to that of the wild-type M. smegmatis Mc^c155 strain by thin layer chromatography (TLC). The apolar and polar lipids from wild-type M. smegmatis and NJS20.1 were extracted and analyzed by one-dimensional and two-dimensional TLC. In contrast to the previous observations in [9], no difference was observed in the apolar fractions of the two strains (unpublished data). However, a new spot was observed in the polar fractions of NJT20.1 (Figure 6). Similar results were noted after [1-14C]-
Figure 7. DNA Binding Properties of Lsr2
(A) EMSA assay of radiolabeled P_iniBAC in the presence of Lsr2. Five fmole (0.7 ng) of P_iniBAC were incubated with the following amounts of Lsr2: lane 1, 0 ng; lane 2, 400 ng; lane 3, 200 ng; lane 4, 100 ng; lane 5, 50 ng; lane 6, 25 ng.
(B) Determination of the Kd of Lsr2-binding activity to P_iniBAC. Five fmole (●) and 50 fmole (○) of radiolabeled P_iniBAC were incubated with the following amounts of Lsr2: 0 ng, 50 ng, 100 ng, 200 ng, 400 ng, and 800 ng; and then analyzed in EMSA assays.
(C) Competition analysis. Fifty fmole of radiolabeled P_iniBAC were incubated with 200 ng of Lsr2 where indicated. Different amounts of either cold P_iniBAC or poly dI-dC were then added as competitors. Lane 1, no Lsr2 (all other lanes contain Lsr2); lane 2, no competitor; lane 3, 7 ng of P_iniBAC; lane 4, 37 ng of P_iniBAC; lane 5, 72 ng of P_iniBAC; lane 6, 7 ng of poly dI-dC; lane 7, 37 ng of poly dI-dC; lane 8, 72 ng of poly dI-dC.
(D) Specificity of DNA binding. Five fmole of radiolabeled P_iniBAC were incubated with 200 ng of Lsr2 where indicated by a “+” followed by treatment with 0.5% SDS or SDS plus 0.1% glutaraldehyde where indicated. Lane 1, radiolabeled P_iniBAC only; lane 2, Lsr2 added; lane 3, SDS only; lane 4, Lsr2 plus SDS; lane 5, SDS plus glutaraldehyde only; lane 6, Lsr2 plus SDS and glutaraldehyde.

Figure 8. Progressive Lsr2 Oligomerization in the Presence of DNA
Lsr2 (1 µg) was incubated with 0.5 µg of P_iniBAC DNA in the presence of 0.1% of glutaraldehyde. Aliquots were analyzed at different time points on a Coomassie blue-stained SDS polyacrylamide gel. Lane 1, Lsr2 alone (control); lane 2, 1 min; lane 3, 2 min; lane 4, 5 min; lane 5, 10 min. Arrows indicate the location of the principal oligomer bands.

acrylate labeling of actively dividing cells. These experiments suggest that this new compound is a glycolipid because it was visualized with orcinol, a reagent for detecting glycolipids, and the compound migrated like a glycolipid [19]. Furthermore, the compound integrated [1-14C]-acetate, indicating that it contains fatty acids. Transposon mutants may be complicated by polar effects, although this phenomenon can usually be controlled for by complementation experiments. We considered the possibility that the discrepancy between our results and those of Chen et al. could have been due to differences in the location of the transposon insertion. However, we obtained identical results when we repeated the lipid analysis with the Δlr2 strain NJS22 (unpublished data).

Lsr2 Is a Small Basic Protein with Histone-Like DNA-Binding Properties
We prepared recombinant Lsr2 and then performed electrophoretic mobility shift assays (EMSAs) to characterize the ability of Lsr2 to specifically bind P_iniBAC. A large mobility shift was observed when Lsr2 was incubated with a 227-bp PCR amplicon of the P_iniBAC (Figure 7A). Titration of Lsr2 against two concentrations of P_iniBAC (5 and 50 fmole) showed a dissociation constant (Kd) of approximately 1 µM (Figure 7B). However, Lsr2 appeared to bind to DNA non-specifically, because a 200-bp PCR amplicon of the M. tuberculosis 16S rRNA gene produced similar mobility shifts and exhibited a similar Kd (unpublished data). Furthermore, competition analysis with unlabeled P_iniBAC and poly dI-dC DNA (Figure 7C) and 1 kb ladder; unpublished data) demonstrated an equal or better ability to compete for Lsr2 binding.

M. tuberculosis Lsr2 has a predicted mass of approximately 12 kDa and a pl of 10.69. These properties suggested that Lsr2 might have features in common with bacterial histone-like proteins [20] even though BLAST and iterative PSI-BLAST searches did not reveal any significant similarities. The mobility shifts observed in the EMSA assays indicated a complex that was much larger than would be expected by the association of a single 12-kDa Lsr2 molecule with its DNA target (Figure 7A). The formation of large protein–DNA complexes has also been reported with histone-like proteins [21–23]. We performed cross-linking studies between Lsr2 and P_iniBAC DNA to test the specificity of the interaction between Lsr2 and DNA and to rule out the possibility that these complexes were caused by electrostatic binding artifacts. Treatment with 0.1% sodium dodecyl sulfate (SDS) caused the Lsr2–DNA complexes to dissociate, resulting in a loss of the original gel shift (Figure 7D). However, the shift was recovered by the addition of 0.1% of glutaraldehyde to the samples prior to SDS treatment. Lsr2 was then incubated with P_iniBAC in the presence of glutaraldehyde for various times (Figure 8). Lsr2 multimers were detectable (in the form of multiple bands) as early as 1 min; longer incubation times produced very large complexes. Similar results were obtained after incubating Lsr2 with 1 kb ladder molecular weight
marker DNA under similar conditions (unpublished data). These results demonstrate that Lsr2 directly interacts with a broad range of DNA sequences, resulting in the formation of large oligomeric complexes.

Histone-like proteins have been reported to preferentially bind supercoiled DNA compared to linear DNA [20,22,24]. We incubated various amounts of Lsr2 with supercoiled and linear pCV125 vector to examine binding preference in an agarose-based gel shift assay. Identical experiments were also performed with a pCV125 vector containing the PiniBAC sequence (pG21898–12) to determine whether Lsr2 preferentially bound to PiniBAC under either of these conditions. A gel shift was only observed in the presence of the supercoiled plasmid. The results were similar whether or not the pCV125 plasmid contained PiniBAC sequences (Figure 9).

Lsr2-Dependent DNA Protection and Inhibition of In Vitro Transcription

Histones and histone-like proteins are typically able to protect DNA from degradation by DNase [20,23]. This property was tested in Lsr2 by first incubating UX174 DNA with Lsr2 and then treating the complex with 0.02 or 1.0 unit of DNase I for 1 min. Lsr2 was inactivated in each sample by treatment with protease K followed by boiling in SDS prior to analysis by gel electrophoresis to prevent a confounding gel shift of the Lsr2-treated DNA. We found that DNase I digested UX174 DNA into small fragments averaging less than 100 bp in size in the absence of Lsr2 pretreatment (longer periods of DNase I digestion completely digested the DNA). In contrast, DNase I activity was substantially inhibited by pretreatment with Lsr2 (Figure 10). Lsr2 pretreatment followed by digestion with 0.02 unit of DNase I produced a wide range of DNA fragments ranging from approximately 150 bp to the size of the supercoiled vector. Lsr2 pretreatment also conferred some protection against the activity of the higher concentration of DNase I, resulting in DNA fragments with an average size of approximately 150 bp (Figure 10), while this same concentration of DNase I completely digested the DNA sample in the absence of Lsr2 pretreatment (unpublished data). Heat-treated Lsr2 retained the ability to inhibit DNase, which is consistent with the known heat stability of histone-like proteins [25,26]. This protective effect could be the result of a histone-like interaction between Lsr2 and the DNA target of DNase. Alternately, Lsr2 could be inhibiting DNase due to a direct interaction between the Lsr2 and DNase proteins. DNase could not be co-eluted with Lsr2 bound to a nickel matrix when this experiment was performed to test for protein–protein interactions. These results suggest that Lsr2 does not interact directly with DNase.

We also tested the ability of Lsr2 to inhibit transcription in vitro as has been reported for other histones and histone-like proteins [27,28]. We used a standard in vitro T7 promoter-
based transcription assay of a pGEM vector for these experiments because Lsr2 did not appear to bind specifically to M. tuberculosis DNA sequences. In the absence of Lsr2 pretreatment, transcription of the pGEM vector produced the expected 1.0- and 2.3-kb mRNA transcripts (imidazole was added to these reactions to control for the presence of imidazole in the buffer containing Lsr2). The expected mRNA transcripts were also present in transcription reactions containing 200 ng of Lsr2; however, 600 ng of Lsr2 completely inhibited transcription (Figure 11). In order to confirm that transcription was not being inhibited by nonspecific effects of an added protein, we repeated these experiments after identical amounts of M. tuberculosis ESAT 6 protein were added to the transcription reaction. In contrast to Lsr2, ESAT 6 did not inhibit transcription (unpublished data).

Effect of Lsr2 on Topoisomerase I–Dependent Supercoil Relaxation

DNA relaxation assays are often used to characterize the ability of histones and histone-like proteins to introduce supercoils into relaxed DNA in the presence of topoisomerase I [7,22]. We relaxed supercoiled ΦX174 DNA with topoisomerase I, then added Lsr2 and measured its ability to re-introduce supercoils. Lsr2 produced a small degree of additional supercoils to the relaxed DNA in this assay, consistent with histone-like activity. A small amount of linear DNA was also produced, suggesting that Lsr2 has nuclease properties (Figure 12A). These results could indicate that Lsr2 has only a modest histone-like ability to introduce supercoils into DNA. However, it was possible that Lsr2 also inhibited the interaction between topoisomerase I and the DNA target, which is also necessary for the introduction of supercoils in this assay. To differentiate between these two possibilities, we repeated the DNA relaxation assay, this time simultaneously adding Lsr2 and topoisomerase I to the supercoiled ΦX174 DNA. We found that topoisomerase I produced substantially less relaxed DNA when it was co-incubated with Lsr2, especially when higher amounts of Lsr2 were used (Figure 12B). Inhibition of topoisomerase I appears to be a novel activity that has not been reported for other bacterial histone-like proteins.

Figure 11. Effect of Lsr2 on In Vitro Transcription

The pGEM plasmid was pre-incubated with different concentrations of Lsr2 where indicated. In vitro transcription was then performed either in transcription buffer or in buffer plus added imidazole (the Lsr2 elution) at the appropriate control concentration. Lane 1, pGEM with 200 ng of Lsr2; lane 2, pGEM with 600 ng of Lsr2; lane 3, pGEM with 40 nM imidazole (control for lane 1); lane 4, pGEM with 120 nM imidazole (control for lane 2).

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Figure 12. Effect of Lsr2 on Topoisomerase I Activity

Different amounts of topoisomerase I were added to 200 ng of supercoiled ΦX174 RT DNA. Lanes 1, 5, and 9, no topoisomerase; lanes 2, 6, and 10, 2.4 units of topoisomerase; lanes 3, 7, and 11, 6 units of topoisomerase; lanes 4, 8, and 12, 12 units of topoisomerase. Lsr2 at 0.1 mg/ml or 0.4 mg/ml was added to the reaction either simultaneously with the topoisomerase or after 30 min incubation with topoisomerase alone. The reaction was then treated with 6% SDS and 4 mg/ml proteinase K analyzed on a 0.7% agarose gel and then stained with ethidium bromide.

(A) Topoisomerase I and Lsr2 were incubated simultaneously with ΦX174.

(B) Lsr2 was added 30 min after the topoisomerase incubation.

L, linearized plasmid; R, relaxed plasmid; S, supercoiled plasmid.

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Discussion

We have shown that Lsr2 is a histone-like protein with broad downregulatory and (to a lesser extent) upregulatory activity in M. smegmatis. The lsr2 gene also appears to regulate the degree of EMB-mediated induction in a large set of genes that are induced in wild-type M. smegmatis by EMB. In M. tuberculosis, lsr2 appears to downregulate antibiotic-mediated induction of the M. tuberculosis iniBAC and effA genes. Our results suggest that lsr2 has a role in regulating the drug tolerance phenotype that is associated with iniA overexpression in M. smegmatis and BCG. Lsr2 is also likely to have other regulatory functions, including those associated with cell wall biosynthesis, transport, and responses to antibiotic treatment.

The lsr2 genes of M. leprae, M. tuberculosis, and M. smegmatis share an unusually high degree of homology (87% identity and 91% similarity for M. leprae compared to M. tuberculosis; 87% identity and 90% similarity for M. smegmatis compared to M. tuberculosis), suggesting an important biological role in these species. Lsr2 was first reported to be one of the major seroreactive proteins in M. leprae patients, and was further characterized as a seroreactive protein in both leprosy and tuberculosis [29,30–32]. Our discovery that Lsr2 binds DNA may explain the high immunoreactivity observed in these prior studies. We postulate that Lsr2 exists as a complex with mycobacterial DNA in extracellular fluid, where it serves as a potent adjuvant by simulating TLR-9 in macrophages and dendritic cells [33]. However, the importance of this immune response in immunopathogenesis or host immunity to tuberculosis or leprosy remains unclear. The lsr2 gene has been previously reported to be induced by a number of stress conditions, including starvation, heat shock, and INH treatment [34–36]. Lsr2 production was also found to be induced in cultures supplemented with iron [37]. The association between induction of lsr2 expression and these stress responses may provide additional clues to its role in cellular regulation.

Histones have been shown to broadly regulate transcription in eukaryotic cells through their influence on chromosomal topology [20]. The histone-like proteins of bacteria represent a diverse group of molecules that share the common property of small size and strong positive charge. Bacterial histone-like proteins have been associated with regulation of various cell stresses or responses to environmental changes [7,38,39]. Some histone-like proteins in Escherichia coli have been shown to directly affect antibiotic resistance by controlling expression of efflux pumps [40]. Disruption of hupA (one of the two genes encoding the HU protein) in E. coli K-12 had recently been shown to cause morphological changes similar to those we observed in NJS20.1 and NJS22 [41]. Very little is known about histone-like proteins in mycobacteria. The M. smegmatis hlp gene encodes a histone-like protein that is induced by cold-shock [42] and anaerobic-induced dormancy [43]. The hlp gene was also found to be important for invasion of M. leprae into peripheral nerves, and it has been hypothesized that it acts as an adhein during mycobacterial infections [44]. MDPI, the M. tuberculosis and BCG homolog of hlp, has recently been shown to bind DNA and inhibit transcription in vitro in a manner similar to that of Lsr2. MDPI is also induced in stationary phase cultures, and appears to participate in the binding of M. tuberculosis to alveolar epithelial cells [45–47]. However, unlike lsr2, MDPI has homologies to other histone-like proteins, such as hlp and the E. coli HU protein; furthermore, the regulatory roles (if any) of MDPI, and other histone-like proteins in M. tuberculosis, are not known.

In addition to its size and pl, Lsr2 shares many properties with other bacterial histone-like proteins. Our microarray studies identified clusters of genes with AT-rich 5-prime untranslated sequences that were induced in the Δlsr2 strain. This finding closely parallels the activity of the histone-like protein H-NS, which transcriptionally silences clusters of laterally acquired genes in Salmonella by binding to AT-rich sequences [11]. We showed that Lsr2 forms large multimeric complexes with DNA (with a preference to supercoiled forms), protects against DNase I treatment, and introduces a modest degree of supercoiling into relaxed plasmids, properties consistent with histone-like proteins. Lsr2 also appears to inhibit in vitro transcription and topoisomerase I. Co-elution studies did not detect any interactions between Lsr2 and DNase, suggesting that Lsr2 exerts its suppressive effect by interacting with DNA rather than by directly inhibiting proteins. Given the other similarities of Lsr2 to histone-like proteins, it is likely that Lsr2 inhibits RNA polymerase and topoisomerase activity by causing topological changes to the DNA targeted by these enzymes. This inhibition may be related to the ability of Lsr2 to form large oligomeric complexes with DNA. It is possible that the activity of Lsr2 is modulated in vivo by other cellular proteins and by local variations in chromosomal sequences; however, this remains to be determined. A PSI-BLAST analysis of the Lsr2 sequence reveals a nuclease motif, which is consistent with the weak nuclease activity that was noted in some of our experiments and may be related to its function. The Lsr2 sequence is unique, exhibiting no significant similarities to any histone-like protein. Thus, Lsr2 represents a novel class of histone-like proteins.

Our discovery that Lsr2 is involved in regulating a subset of INH- and EMB-inducible genes suggests at least one significant function. Investigations of the cellular responses to antibiotic treatments (as distinct from investigations of antibiotic resistance mechanisms) are in their infancy. Although deletion of the histone-like protein gene hns in E. coli was found to de-repress expression of multi-drug transporters related to TolC and confer multi-drug resistance [40], this work represents the first investigation to our knowledge of the regulation of antibiotic-induced genes in M. tuberculosis. Studies of cellular responses to antibiotics may be crucial to understanding the mechanisms by which bacteria survive or die in the presence of antibiotics. For example, we previously demonstrated that the iniBAC genes are induced by INH, EMB, and a number of other antibiotics that act by inhibiting cell wall biosynthesis in M. tuberculosis [8]. Induction of iniA was shown to confer multi-drug resistance through the action of a multi-drug resistance-like pump [3]. Antibiotic tolerance can occur through other mechanisms such as overproduction of various inhibitors, enzymes, and regulatory proteins in non-mycobacterial bacteria [4,5,48–50]. It is possible that a multi-functional lsr2 also regulates these and other pathways in mycobacteria.

Deletion analysis of lsr2 in M. tuberculosis would be particularly useful for studies of its function. Unfortunately, we have been unable to delete lsr2 from this species, although deletion was easily accomplished in M. smegmatis. The lsr2
gene may be essential in *M. tuberculosis*, *lsr2* was suggested to be essential by Himar1-based transposon mutagenesis [13], and all of the *lsr2* transposon mutants characterized by McAdam et al. [14] contained insertions at the extreme 3′-prime end of the gene where the transposon would be unlikely to affect functional capacity. If confirmed by future studies, the finding that this gene is essential is consistent with our hypothesis that *lsr2* regulates important cellular pathways in *M. tuberculosis*.

The *lsr2* gene has been found to be essential for biofilm formation in *M. smegmatis*. This study also showed that an *M. smegmatis* *lsr2* transposon mutant had altered colony morphology and contained two previously unidentified apolar lipids that were novel mycolate-containing compounds [9]. We found the same altered colony morphology but did not detect any novel apolar lipids in our analysis of the *M. smegmatis* (*lsr2::Tn5370*) strain NJS20.1 or in the Δ*lsr2* strain NJS22. However, we did detect a new compound, possibly a glycolipid, in the polar fraction of both NJS20.1 and NJS22. The dissimilarity between these two lipid analyses could be due to a difference in the way the strains were grown or harvested for the TLC analysis. Despite the apparent contradiction between the two studies, both investigations suggest that *lsr2* is involved in regulating a wide range of cellular processes.

In summary, *lsr2* encodes a histone-like DNA-binding protein that appears to be essential for controlling responses to certain types of antibiotic stress. *Lsr2* has also been linked to other types of stress responses and other cellular functions in mycobacteria. An improved understanding of the role of *lsr2* and of the stress responses associated with this gene may provide important insights into the mechanisms of action of antibiotics and the way that mycobacteria adapt to certain types of stresses such as antibiotic treatment. This knowledge could in turn be used to design more effective antibiotic treatments for both drug-susceptible and drug-resistant *M. tuberculosis*.

**Materials and Methods**

**Bacterial strains and culture conditions.** *E. coli* DH5α was the host for all plasmid constructions. Experiments with *M. smegmatis* either used Mc^155^ or, in the case of the transposon mutagenesis experiments, NJS20, a Mc^155^ strain containing the pG21898–12 plasmid integrated into Mc^155^ chromosome at attP (Table 1) [8]. The pG21898–12 plasmid is a reporter construct that contains the *M. tuberculosis* P*inhA*-lacz fused to lacZ [8]. Experiments with *M. tuberculosis* used strain H37Rv. *E. coli* was cultured at 37°C in Luria-Bertani medium with the addition of hygromycin B (200 μg/ml; Sigma, http://www.sigmaaldrich.com) or kanamycin (40 μg/ml; Sigma) where appropriate. *M. smegmatis* strains were grown at 37°C on a rotary shaker in Middlebrook 7H9 medium (Difco, http://www.vgdusa.com/Difco.htm) containing 0.05% Tween 80, 0.02% glycerol, 10% ADC (Sigma) [51], and 25 μg/ml kanamycin or 40 μg/ml apramycin as appropriate. Transposon mutants of NJS20 were cultured in the presence of hygromycin B (50 μg/ml). *M. tuberculosis* strains were cultured at 37°C on a rotary shaker in Middlebrook 7H9 medium (Difco) containing 0.05% Tween 80, 0.02% glycerol, and 10% ADC with 12.5 μg/ml kanamycin added as appropriate.

**Plasmid construction.** The complete *lsr2* gene (nucleotides 4040981–4041319) was amplified by PCR from H37Rv chromosomal DNA using primers pMV261-*lsr2*F and pMV261-*lsr2*R (Table 1), digested with PvuII and ClaI, and then cloned into pMV261 [52] (which encodes for kanamycin resistance) to create pMV261:*lsr2*, or into pMMP167 [53] (which encodes for apramycin resistance) at the PstI/ClaI sites to create pMMP167:*lsr2* (Table 1). *M. tuberculosis* *lsr2* ORF (nucleotides 4040981–4041319) was inserted into the Ndel/XhoI sites of pET-30, creating pET::*lsr2* and then into pET-30, creating pET-LSR2. The pET:*lsr2* plasmid was transferred into BL-21 *E. coli* competent cells. Plasmids pCV125, pG21898–12, and pBluescript have been described elsewhere [8].

**Transposon mutagenesis.** Transposon mutagenesis was performed in the *M. smegmatis* iniBAC promoter reporter strain NJS20 as described [54] using the minitransposon vector pJSC84, which contains inverted repeats flanking a hygromycin cassette [54]. The construct was packaged in a TM4 temperature-sensitive phage, and transfected into NJS20. Cells were grown in 7H9 to mid-log phase, prewarmed to the non-permissive temperature of 37°C, and then mixed with 10^11^ pfu/ml (multiplicity of infection 10). The cell–phage mixture was incubated at the non-permissive temperature of 30°C for 30 min, plated on 7H10 agar containing IPTG and β-galactosidase, and then incubated at 37°C for 2-3 d.

**Creating an unmarked deletion of *lsr2* in *M. smegmatis*.** Strain NJS22, an *M. smegmatis* strain containing a complete unmarked deletion of *lsr2*, was created using the sacB counter selection method as described previously [55]. Briefly, DNA sequences from 615414 to 615817 and from 615610 to 6157718 in *M. smegmatis* Mc^155^ were PCR amplified using primer pairs F1-lsr2KO - R13-lsr2KO and F2-lsr2KO - R2 lsr2KO, respectively (Table 1), and cloned into the p2NIL vector [55], followed by insertion of a Pacl cassette containing sacB and lacZ. Blue colonies containing single crossover events were identified on X-gal/kanamycin media. Double crossover events from these blue colonies were selected on 2% sucrose/X-gal media. Deletion mutants were confirmed by real-time PCR for the *lsr2* gene using primers Flsr2SC and Rlsr2SC (Table 1).

**β-galactosidase assays.** Assays were performed as described [8] using o-nitrophenyl-β-D-galactopyranoside (4 mg/ml; Sigma) to detect the presence of β-galactosidase activity. β-galactosidase units were calculated using the formula 1,000 × OD_{420}/time (minutes) × 0.5 × OD_{500}.

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**Table 2. Genes Upregulated in All Three Comparisons**

| Locus         | Gene Annotation                        | Function   |
|---------------|----------------------------------------|------------|
| MSMEG_255     | Phosphoenolpyruvate carboxykinase       | Metabolism |
| MSMEG_695     | Ionized inducible protein InIa           | Transport  |
| MSMEG_1064    | Phosphate/sulfate permease              | Transport  |
| MSMEG_155     | Conserved hypothetical protein          | Unknown    |
| MSMEG_2288    | Conserved hypothetical protein          | Unknown    |
| MSMEG_3199    | Quinolinate synthetase complex, A subunit | Metabolism |
| MSMEG_3564    | Bacteriorhodopsin                      | Transport  |
| MSMEG_4342    | Metallo-beta-lactamase family protein   | Resistance |
| MSMEG_6201    | Transglycosylase                       | Cell envelope |
| MSMEG_6904    | Mucin-inositol-1-phosphate synthase     | Cell envelope |
| MSMEG_6919    | Proline-rich 28-kDa antigen             |            |

Comparison 1: expression of NJS20 compared to NJS22.
Comparison 2: expression of NJS20 compared to NJS20 cultured in EMB.
Comparison 3: expression of NJS20 compared to NJS22 both cultured in EMB.

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Generation of probes for microarray experiments. cDNA probes for microarray experiments were generated as previously described [56]. One microgram of mRNA in a mixture containing 6 µg of random hexamers (Invitrogen, http://www.invitrogen.com), 0.01 M dithiothreitol, an aminooethyl-deoxycytoside triphosphate mixture containing 25 mM each dATP, dCTP, dGTP, and dTTP, and 10 µM dUTP in 60 µl of hybridization solution (20 mM Tris-Cl (pH 8.0), 0.5 M NaCl) was added to 1 unit of T7 RNA polymerase (Promega, http://www.promega.com) for 1 h at 37 °C followed by incubation at 65 °C for 15 min. Unincorporated dUTP was removed with a Minelute column (Qiagen, http://www.qiagen.com). The probe was eluted with a phosphate elution buffer (4 mM KPO4 [pH 8.5], in ultrapure water), dried, and resuspended in 0.1 M sodium carbonate buffer (pH 9.0). To couple the amino-allyl cDNA with fluorescent labels Cy3 or Cy5 (Amersham Pharmacia Biotech) was added at room temperature for 2 h. Uncoupled label was removed using the Qiagen Minelute PCR purification.

Microarray hybridization, scanning, image analysis, normalization, and analysis. Microarray studies of each condition were performed using three separate RNA samples obtained from separate cultures using a dye-flip protocol (two microarrays for each RNA sample). Epoxy- or aminosiline-coated slides were prehybridized in 5x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (Invitrogen), 0.1% SDS, and 1% albumin at 37 °C for 60 min, then were washed at room temperature with distilled water, dipped in isopropanol, and spun dry. Equal volumes of the appropriate Cy3- and Cy5-labeled probes were combined, dried, and then resuspended in a solution of 40% formamide, 5x SSC, and 0.1% SDS. Resuspended probes were denatured to 95 °C prior to hybridization. The probe mixture was applied to the prehybridized slides and hybridized overnight at 42 °C. Hybridized slides were washed sequentially in solutions of 1x SSC and 0.2% SDS, 0.1x SSC and 0.2% SDS, and 0.1x SSC at room temperature, then dried, and scanned with an Axon GenePix 4000 scanner (http://www.axon-moleculardeisves.com). Individual TIF images from each channel were analyzed with TIGR Spotfinder (http://www.tm4.org). Microarray data were normalized by Iterative Log Normalization using TIGR GeneSpring (www.tigr.org) for 1 h at room temperature. The unbound Lsr2 was removed by washing the matrix three times with 0.1 M PBS, 20 mM MgCl2, and 20 mM NaCl. DNA from Lsr2 (5 µg; New England Biolabs, http://www.neb.com) was added to the nickel matrix-Lsr2 and incubated overnight at 4 °C. After washing with 0.1 M PBS, 20 mM MgCl2, and 20 mM NaCl, Lsr2 was eluted from the nickel matrix using 0.1 M PBS, 20 mM MgCl2, 20 mM NaCl, and 20 mM imidazole. Eluted proteins were assessed in each fraction by Coomassie blue-stained SDS-PAGE.

In vitro transcription. Briefly, 0.5 µg of pGEM was incubated with either control imidazole buffer or either 200 or 600 ng of Lsr2 in a final volume of 20 µl, followed by in vitro transcription according to the manufacturer’s recommendation using the Riboprobe in vitro Transcription Kit (Roche Diagnostics). RNA fractions were separated by gel electrophoresis and visualized under UV light.

Gene expression in the presence and absence of INH. Total RNA was extracted from M. tuberculosis strains. cDNA synthesis and quantitative PCR with molecular beacons were performed as previously described [57]. The molecular beacons and primers used to study expression of kanR and inhA were described previously [57], and the molecular beacons and primers used to study expression of lsr2, mbb, mbA, mbC, and ebpA are shown in Table 1.

Lipid extraction. M. smegmatis strains (50 ml) were grown in Mueller-Hinton broth (Difco) at 37 °C up to late log phase. The cultures were centrifuged, and the pellets were washed once with PBS buffer. After discarding the supernatants, the cell pellets were lyophilized. The dried pellets were treated as described previously [19]. Briefly, the cell pellet (0.2 g) was resuspended in methanol/0.3% aqueous NaCl solution (2 ml; 10/1, v/v) and the suspension was extracted twice with petroleum ether (1 ml) for 15 min at room temperature. The petroleum ether phases were combined and dried under nitrogen to yield the apolar lipid fraction, which was resuspended in dichloromethane. The methanol/saline fraction was heated at 100 °C for 5 min, cooled, and treated with chloroform/methanol (90/10, v/v) for 1 h followed by chloroform/methanol (90/10, v/v) for 0.5 h. The combined chloroform/methanol phases were washed three times with a 2 M sodium chloride solution. The chloroform/methanol phases were dried under nitrogen and reconstituted with 0.5 ml of methanol/water (9/1, v/v). The chloroform/methanol extract was evaporated to dryness under nitrogen. The chloroform/methanol extract was dissolved in 200 µl of chloroform/methanol (90/10, v/v) and analyzed on thin layer chromatography (TLC) plates.

Electrophoretic mobility shift assay. The 227-hp promoter region of P BAC (nucleotides 409579–409560) was amplified by PCR reaction using 32P-dCTP in the reaction. Radiolabeled P BAC, poly dl-dC, or 1 kb ladder was loaded on 4% polyacrylamide gel and allowed to run for 20 min on ice in a 10-ml reaction cocktail containing 20 mM Tris-HCl (pH 7.0), 0.01% BSA, 2 mM DTT, and 10 mM NaCl. Protein-DNA complexes were resolved by electrophoresis through a 5% polyacrylamide gel for 1 h at 4 °C and then examined by autoradiography.

Protein–protein cross-linking. Different amounts of recombinant Lsr2 were incubated with P mub, DNA in a mixture containing 20 mM Tris-HCl (pH7.0), 0.01% BSA, 2 mM DTT, and 10 mM NaCl. Cross-linking was performed by the addition of 0.1% of glutaraldehyde (Sigma) to the mix. The mix was incubated and loaded onto a 10% polyacrylamide gel at different time points.

DNA1 protection. 4X174 (Promega, http://www.promega.com) plasmid DNA (0.5 µg) was incubated for 20 min on ice with or without Lsr2 (200 ng) in a final volume of 50 µl. Either 1 unit of DNAse I (New England Biolabs, http://www.neb.com) was then added, and the mixture was incubated at 37 °C for 1 min. DNAse was inactivated by incubating at 75 °C for 15 min. The samples were then treated with 6% SDS and 4 mg/ml of protease K for 30 min at 37 °C, and then analyzed on a 1% agarose gel.

Conclusion studies. Recombinant Lsr2 (1 µg) was incubated with a nickel Sepharose matrix (GE Healthcare, http://www.genhealthcare.com) for 1 h at room temperature. The bound Lsr2 was removed by washing the matrix three times with 0.1 M PBS, 20 mM MgCl2, and 20 mM NaCl. DNAse I (5 µg; New England Biolabs, http://www.neb.com) was added to the nickel matrix-Lsr2 and incubated overnight at 4 °C. After washing with 0.1 M PBS, 20 mM MgCl2, and 20 mM NaCl, Lsr2 was eluted from the nickel matrix using 0.1 M PBS, 20 mM MgCl2, 20 mM NaCl, and 20 mM imidazole. Eluted proteins were assessed in each fraction by Coomassie blue-stained SDS-PAGE.

Regulation of Gene Expression by Lsr2

The following solvent systems were used to run one-dimensional TLC using silica gel 60F-254 TLC plates (Alltech, http://www.alltech.com). The following solvent systems were used to run one-dimensional TLC: hexane/ethyl acetate (91/1, v/v); hexane/ethyl acetate (11/1, v/v); ethyl acetate; chloroform/methanol (95/5, v/v); chloroform/methanol (90/10, v/v); and chloroform/methanol/water (60/30/6, v/v). For two-dimensional TLC, the solvent systems were used as follows: either first dimension, chloroform/methanol/water (100/40/8, v/v), second dimension, chloroform/methanol.
acetone/methanol/water (50:60:2, 5/3, v/v/v/v); or first dimension, chloroform/methanol/water (60:50, v/v), second dimension, chloroform/acetic acid/methanol/water (40/25/0.6, v/v/v/v). The lipids were visualized by spraying with a 10% sulfuric acid solution in ethanol or with an orcinol solution (Altech).

**Radiolabeling of lipids with [1-14C]-acetate.** The M. smegmatis strains were grown in Middlebrook 7H9 supplemented with 0.2% glucose, 10% ADS enrichment, and 0.2% Tween 80 to log phase (OD_{600} = 0.8), and labeled with [1-14C]-acetate (15 μCi) for 1 h. After centrifugation, the lipids were extracted as described above.

**Supporting Information**

**Table S1.** Genes Upregulated

Found at doi:10.1371/journal.ppat.0030087.s001 (1.4 MB XLS).

**Table S2.** Genes Downregulated

Found at doi:10.1371/journal.ppat.0030087.s002 (161 KB XLS).

**Table S3.** Genes Downregulated

Found at doi:10.1371/journal.ppat.0030087.s003 (114 KB XLS).

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