

CLONING, EXPRESSION AND CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* *SIRR*

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Abstract. Identification of new drug targets is important for the improvement of chemotherapy for tuberculosis treatment. Metal-associated gene products are candidates for novel drug development. A *Mycobacterium tuberculosis* (MTB) *sirR*-encoded protein has been proposed, but the function of MTB SirR has not yet been elucidated. Bioinformatics analysis revealed that MTB SirR contains iron binding domains with 34%-59% similarity to previously described metal-dependent gene regulators and that the gene lies in *Rv2787-sirR* operon. RT-PCR revealed that the *Rv2787-sirR* operon is transcribed a single bicistronic mRNA. Heterologous expression, purification and characterization of recombinant MTB His-tagged SirR demonstrated a 25 kDa protein (by SDS-PAGE and immunoblotting) that exists as a dimer (native PAGE). Based on electrophoretic mobility shift assay, MTB SirR bound a cis element located at -85 bp upstream of its operon. As *Rv2787-sirR* operon is unique only to MTB (and *M. bovis*), further studies on its regulation and other functions of the encoded proteins should provide leads towards the discovery of novel anti-TB drugs.

Keywords: *Mycobacterium tuberculosis*, iron-dependent regulator, *Rv2787-sirR* operon, SirR protein

INTRODUCTION

Mycobacterium tuberculosis (MTB) is the causative agent of tuberculosis (TB), a disease that continues to be a major public health problem, causing approximately 2 million deaths each year worldwide (WHO, 2010). Approximately one-third of the world's population is latently in-

fectured with MTB (Young *et al*, 2009). To date, there is no drug that effectively kills dormant bacilli.

Metal ion-associated proteins of MTB are good targets for TB treatments, because metal ions are crucial for survival in the host and maintenance of the infection (Clemens and Horwitz, 1996; Boelaert *et al*, 2007; Lucarelli *et al*, 2008; Reddy *et al*, 2012). MTB possesses several putative metal-responsive transcriptional regulatory genes, including *ideR*, *furA*, *furB*, and *sirR*. IdeR is a metal-dependent regulator involved in various physiological functions, especially that of iron storage (Rodriguez *et al*, 1999), and FurA and FurB

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act as negative regulators of *katG* (Zahrt *et al*, 2001), encoding a zinc-dependent regulator of bacterial zinc uptake (Lucarelli *et al*, 2007).

MTB *sirR* has been suggested to encode a 25 kDa iron-dependent dimeric regulator (Saha *et al*, 2009). In *Staphylococcus epidermidis*, *sirR* has a 645-bp open reading frame, encoding a 25 kDa polypeptide characterized as an iron-dependent regulator and is located upstream of *sitABC* operon, which encodes a putative ABC transporter (Hill *et al*, 1998). This gene is a homolog of *Corynebacterium diphtheriae dtxR*, a well studied iron-responsive gene in gram-positive bacteria (Kunkle and Schmitt, 2003). However, studies of *sirR* in MTB are rare, and there has been only one study in MTB (Saha *et al*, 2009). Therefore, further characterization of MTB *sirR* is needed.

Bioinformatics analysis suggests that *Rv2787* and *sirR* form an operon and SirR in MTB was annotated as an iron-dependent regulatory protein (Cole *et al*, 2001). It is known that regulators are capable of controlling genes within their own operons (Namwat *et al*, 2001; Zahrt *et al*, 2001). Therefore, we hypothesized that MTB SirR might have similar properties. The aims of this study were to characterize the *Rv2787-sirR* operon and determine whether SirR is able to bind this operon. Characterization of MTB SirR could lead to the discovery of a new drug target for TB therapy.

MATERIALS AND METHODS

Bioinformatics analysis

The *sirR* operon and binding sites were analyzed using mycoperonDB database (Ranjan *et al*, 2006) and FGENESB program (<http://www.softberry.com>), respectively. A similarity search was per-

formed by comparing regions upstream of the *Rv2787-sirR* operon present in the database using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A multiple sequence alignment between *sirR* and other homologous genes was performed using the ClustalW program (<http://www.ebi.ac.uk/clustalw/>). Conserved domains of MTB SirR were analyzed using the Pfam program (<http://pfam.sanger.ac.uk/>). The molecular weight and pI of SirR were calculated using the Compute pI/Mw tool from the Swiss Institute of Bioinformatics website (ExpASY; <http://www.expasy.org/>).

Bacterial strains and vectors

MTB H37Rv strain was grown in Middlebrook 7H9 (Difco, Sparks, MD) liquid medium supplemented with 0.2% glycerol and 0.05% Tween 80 at 37°C for 14 days. *Escherichia coli* BL21 (DE3) and *E. coli* XL1-Blue strains were cultured in Luria-Bertani (LB) broth (Bertani, 1951). Plasmid pET-32b (+) (Novagen, Darmstadt, Germany) was used as a vector. The broth used to culture *E. coli* harboring plasmid vectors was supplemented with 50 µg/ml ampicillin.

Cloning of recombinant MTB *sirR*

Chromosomal MTB DNA was isolated as previously described (van Soolingen *et al*, 1994). In brief, bacterial cells were sequentially treated with lysozyme, RNase A, SDS, and proteinase K DNA was extracted twice using phenol-chloroform and isoamyl alcohol solutions, and stored at -70°C until used.

A 686-bp region containing MTB *sirR* was amplified from MTB H37Rv chromosomal DNA using two sets of primers bearing different restriction sites for the construction of a histidine (His)- and a thioredoxin-tagged SirR. In order to construct the His-tagged protein, primer pair

(5'-AGCCATATGGTGAGGGCTGACGAG-3') and (5'-ATTGCGGCCGCGCTCACCACCCAGAT-3'), containing *NdeI* and *NotI* restriction sites (underlined), respectively, were used; and for construction of the thioredoxin-tagged protein, the primer pair was (5'-ATACCATGGTGAGGGCTGACGAGG-3') and (5'-ATTGCGGCCGCTCAGCTCACCACCA-3'), bearing *NcoI* and *NotI* restriction sites (underlined), respectively. PCR thermocycling (using a C1000™ Thermal Cycler; Bio-Rad, Hercules, CA) was performed as follows: 25 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute.

The two 686-bp amplicons were digested with their respective restriction enzymes (as described above), ligated with their compatibly digested pET32b (+) plasmids and used to transform competent *E. coli* XL1-Blue cells (Hanahan, 1983). Transformants were selected by plating on LB agar supplemented with ampicillin (25 µg/ml).

Heterologous expression and purification of recombinant MTB *sirR* proteins

Transformed *E. coli* BL21 (DE3) cells were cultured in LB broth containing ampicillin (25 µg/ml) at 37°C until an OD_{600 nm} of 0.6. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added and the cultures were incubated for an additional 4 hours. Bacterial cells were harvested by centrifugation (7,000g at 4°C for 15 minutes), washed with 50 mM Tris-HCl (pH 7), and resuspended in lysis buffer (0.05 M Tris-HCl, pH 7 containing 0.2 M KCl, 20% v/v glycerol, 5 mM dithiothreitol and 0.5 mM p-amidinophenylmethanesulfonyl fluoride). Following cell disruption via sonication (Soniprep 150, MSE, London, UK) for 10 seconds in an ice bath, the suspension was centrifuged (10,000g at 4°C for 15 minutes).

Supernatant containing crude His- and thioredoxin-tagged (also His-tagged) *SirR* proteins were loaded onto a Ni-NTA column (HisTrap™ HP, Amersham Biosciences, Uppsala, Sweden). After the column was washed with binding buffer [100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM (NH₄)₂ SO₄, 5 mM dithiothreitol, 1% w/v Tween 20, 150 mM KCl], the bound proteins were eluted with elution buffer (0.02 M sodium phosphate, 1 M NH₄Cl, pH 7.2). The eluates were analyzed by 12.5% SDS- and native PAGE. The fractions containing recombinant *SirR* proteins (identified by SDS-PAGE) were pooled and dialyzed against phosphate-buffered saline (PBS) overnight at 4°C.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from MTB using Trizol RNA isolation buffer (Invitrogen, Carlsbad, CA) with sonication according to the manufacturer's protocol. Primers used to amplify the five cDNA targets (70 bp and 275 bp fragments in the region upstream of *Rv2787-sirR* operon, and 274 bp, 121 bp, and 537 bp fragments in the regions within the *Rv2787-sirR* operon) (Fig 1) are listed in Table 1. RT-PCR was performed using SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA) and C1000™ Thermal Cycler (Bio-Rad, Hercules, CA) as follows: incubation at 60°C for 15 minutes; 95°C for 5 minutes; 35 cycles of 96°C for 45 seconds, 53°C for 45 seconds, and 72°C for 1 minute; and a final 7 minutes at 72°C. The amplicons were analyzed using agarose gel-electrophoresis and ethidium bromide staining.

Immunoblot analysis

Purified recombinant *SirR* was subjected to 12% SDS-PAGE and transferred onto a nitrocellulose membrane. After

incubating with 3% bovine serum albumin (BSA) in 50 mM PBS, the membrane was washed with PBS-0.05% Tween 20 and incubated for 1 hour with rabbit anti-histidine polyclonal antibodies (GenScript, Piscataway, NJ). After washing with PBS-Tween 20, the membrane was incubated with horseradish peroxidase-conjugated goat secondary antibodies (GenScript, Piscataway, NJ) at 37°C for 1 hour, washed with PBS-Tween 20, and the positive signals were developed using diaminobenzidine and H₂O₂.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (Hamoen *et al*, 1998; Rodriguez *et al*, 1999). The potential SirR binding sites (at 70 bp and 275 bp upstream of *Rv2787-sirR* operon and at 80 bp within the *Rv2787-sirR* intergenic region) were amplified using the primers listed in Table 1. The PCR conditions consisted of heating at 95°C for 5 minutes, followed by 35 cycles of 30 seconds at 96°C, 60 seconds at 58°C, and 2 minutes at 72°C, with a final 7 minute heating at 72°C. EMSA was performed using a digoxigenin (DIG) gel shift kit (Roche Applied Science, Basel, Switzerland). In brief, the PCR amplicons were mixed with labeling buffer (1 M potassium cacodylate, 0.125 M Tris-HCl pH 6.6, 1.25 mg/ml BSA), 5 mM CoCl₂ solution, 0.05 mM DIG-11-ddUTP solution, and 1 ml of 20 U terminal transferase. The mixture was incubated for 15 minutes at 37°C and placed on ice; the mixture was then precipitated with 60 ml of ethanol at -70°C for 30 minutes. After centrifugation at 14,000 rpm at 4°C for 15 minutes, the pellet was washed with 500 µl of 70% ethanol, dried, and dissolved in TEN buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 M NaCl). The optimal amount of labeled PCR products (3.85

pmol/µl) was mixed on ice with the crude and purified His-tagged SirR proteins (at 3, 6, 9 and 12 µg) and binding buffer (100 mM HEPES pH 7.6, 5 mM EDTA, 50 mM [NH₄]₂SO₄, 5 mM DTT, 1% (w/v) Tween 20, 150 mM KCl) (Rodriguez *et al*, 1999) in 20 µl of total volume. Positive and negative controls from the reagent kit, a reaction without SirR, and reactions containing competitive sequences (unlabeled PCR products for each particular sequence) were used as controls. Nonspecific binding was inhibited by the addition of 1 µg of poly d(I-C) and 1 µg of poly L-lysine. After incubation for 30 minutes at room temperature, the mixtures were analyzed by 6% native PAGE. The oligonucleotide-protein complexes were blotted onto nylon membranes and digoxigenin-labeled probes were detected by the addition of anti-DIG Fab fragments conjugated to alkaline phosphatase and chloro-5-substituted adamantyl-1,2-dioxetane phosphate (CSPD) substrate (Roche Applied Science, Basel, Switzerland). The chemiluminescent signals were detected by autoradiography.

RESULTS

Bioinformatics analysis of MTB *sirR*

MTB *sirR* (NCBI accession no. NC000962) contains 687 nucleotides and encodes a polypeptide with 228 amino acids, with a calculated molecular weight and pI of 24.95 kDa and 5.07, respectively (Expasy web-based program). Conserved domains of SirR were analyzed using Pfam software. Several conserved domains in SirR included an iron-dependent repressor domain, a helix-turn-helix diphtheria toxin repressor (DTXR), a ferrous iron transport protein A (FeoA) domain, and a transcriptional repressor C-terminal domain. BLAST analysis and multiple

sequence alignment revealed 34%-59% similarity between the amino acid sequences of MTB SirR and other iron-dependent repressors from various bacterial species, highest similarity being the iron-dependent repressor of *Corynebacterium glutamicum* ATCC 13032 (Jakubovics *et al*, 2000; Ng *et al*, 2000; Zhang *et al*, 2003; Baliga *et al*, 2004; Monteiro-Vitorello *et al*, 2004; De Zoysa *et al*, 2005). Multiple sequence alignment of the deduced amino acid sequences of these genes revealed moderate similarities with several highly conserved regions of MTB SirR, which suggests that these conserved residues are functionally important.

Rv2787 and *sirR* transcription and transcription start sites

Based on information from the mycoperoDB database and FGENESB program, an operon comprising *Rv2787* and *sirR* located downstream was predicted (Ranjan *et al*, 2006) and both genes are bicistronically transcribed (Fig 1). This prediction was confirmed using RT-PCR with primers that covered both *Rv2787* and *sirR*. The size of the transcribed RNA target was as expected (537 bp), indicating the co-existence of both genes in the same mRNA (Fig 2). Bands of the expected sizes for both *Rv2787* (274 bp) and *sirR* (121 bp) were also amplified in separate reactions.

The transcriptional start site (TSS) of *Rv2787-sirR* operon was demonstrated using RT-PCR with primers specific to the region located -70 bp upstream of *Rv2787* start codon, but not using primers specific to the region located -275 bp upstream (Fig 2).

Expression and purification of SirR proteins

Both His- and His/thioredoxin-tagged SirR proteins were heterologously expressed in *E. coli* BL21 (DE3) and affinity

Table 1

Primer used for amplification of cDNA and DNA targets of *M. tuberculosis*.

Primer name	Primer sequence
<i>a</i>	CTCATCAGACCCACTCCCT
<i>b</i>	GCATACCGAACGATTGGTC
<i>c</i>	CATCGTTTCGATCCAGGTTT
<i>d</i>	GCACACTGTGGTTGTTCTC
<i>e</i>	GAGTCTAGATACCGGCGTG
<i>f</i>	GGATGACGGTTTGGTCGAG
<i>g</i>	GCGAGCTTGC GAATGGACTC
<i>h</i>	GACTATCTGAAGGTCATCTG
<i>i</i>	AATCCGTCGGGATTCGT

Primers *b* and *a* were used to amplify 70 bp fragment upstream of the *Rv2787-sirR* operon, *i* and *a* to amplify 275 fragment upstream of *Rv2787-sirR* operon, *d* and *c* to amplify *Rv2787* (274 bp fragment), *f* and *e* to amplify intergenic region (80 bp fragment), *h* and *g* to amplify *sirR* gene (121 bp fragment), and *d* and *g* to amplify both *Rv2787* and *sirR* together (537 bp fragment).

purified. The molecular weights of the His- and His/thioredoxin-tagged SirR proteins corresponded with the calculated molecular weight of 25 and 42 kDa, respectively (Fig 3A and B).

Native PAGE analysis of the purified recombinant SirR proteins revealed a putative dimer based on the apparent molecular weights (50 and 84 kDa) (Fig 3C). The successful production of recombinant His-tagged SirR was confirmed immunoblotting with anti-histidine polyclonal antibodies (Fig 3D). Sequencing of the recombinant plasmid also confirmed correct insertion of MTB *sirR* (data not shown).

Identification of MTB SirR putative DNA binding sites

In order to identify DNA binding sites of SirR, EMSA was performed using a 275 bp DIG-labeled DNA fragment located

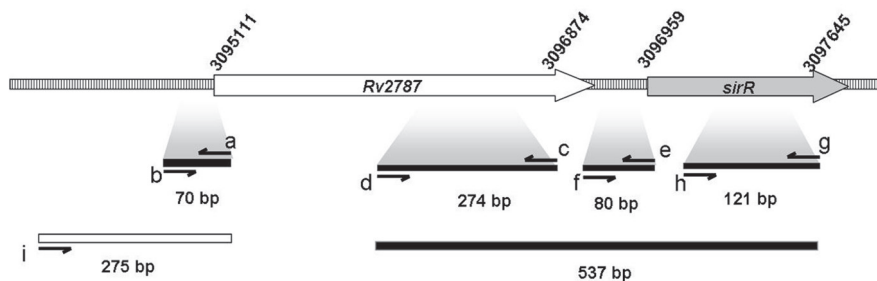


Fig 1—Schematic diagram of the MTB *Rv2787-sirR* operon and primer sites for amplification of target sequences. White bar: target sequence for assay of both transcription and SirR binding; black bars: target sequences for transcription assays. Numbers in italics above *Rv2787-sirR* operon indicate genome positions within the MTB H37Rv strain. Primers *a-i* refer to the names of the primers used to amplify the various fragments (Table1).

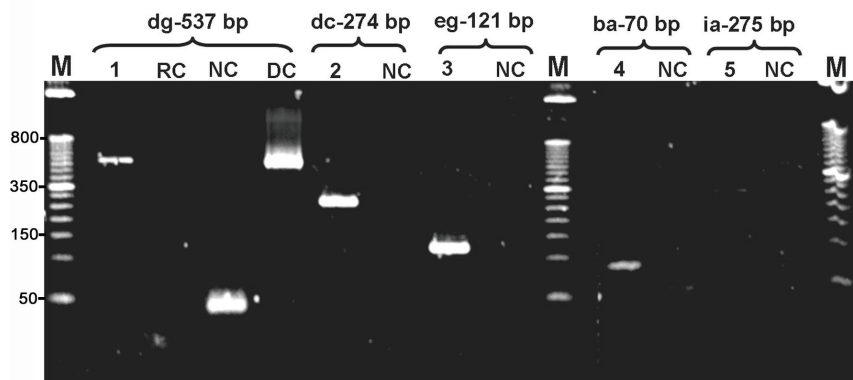


Fig 2—RT-PCR amplification of the MTB *Rv2787-sirR* operon transcript. Experimental protocols are described in Materials and Methods. Lane M: 50 bp DNA ladder (Invitrogen); lane RC: reagent control without template; lane NC: negative control without cDNA; lane DC: reaction with control DNA template; lane1: amplicon using primers *d* and *g* (dg-537 bp); lane 2: amplicon using primers *d* and *c* (dc-274 bp); lane 3: amplicon using primers *e* and *g* (eg-121 bp); lane 4: amplicon using primers *b* and *a* (ba-70 bp); lane 5: amplicon using primers *i* and *a* (ia-275 bp). Primers *a-i* refer to the names of the primers used to amplify the various fragments (Table1).

upstream of *Rv2787* and a DIG-labeled 80 bp fragment located in the intergenic region between *Rv2787* and *sirR*. Crude and purified His-tagged SirR proteins (6, 9 and 12 μ g) were mixed with the labeled DNA probes. Retardation (shift) in gel mobility of the 275-bp (but not 80-bp) labeled DNA probe was apparent when 12

μ g of purified His-tagged SirR was present in the mixture (Fig 4). A more marked shift, but with decreases in band intensity and smearing, was observed in the presence of unpurified His-tagged SirR. The relevant negative controls showed no band shift. Kit positive and negative controls were also performed (Fig 4).

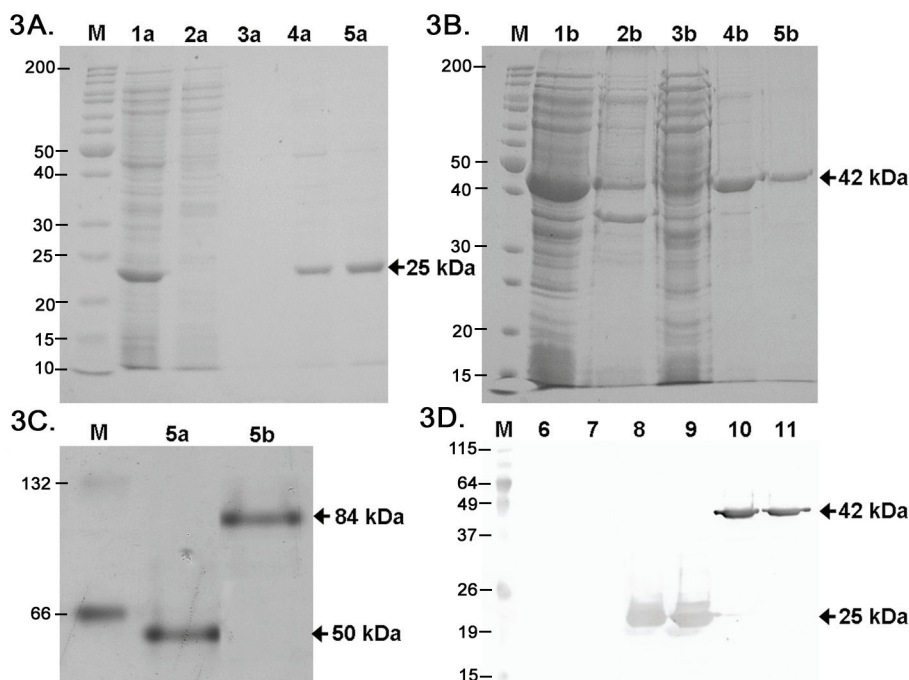


Fig 3—Heterologous expression and purification of recombinant MTB SirR proteins. Recombinant *sirR* containing vector was transformed into *E. coli* BL21 (DE3) cells, induced by IPTG and subsequently culture. Supernatants were collected after cell lysis and purified by HisTrapTM HP column affinity (Amersham Biosciences, Sweden). The eluates were analyzed by PAGE and immunoblotting. A. SDS-PAGE of His-tagged SirR. B. SDS-PAGE of His/thioredoxin-tagged SirR. C. Native PAGE of His- and His/thioredoxin-tagged SirR proteins. D. Immunoblot of His- and His/thioredoxin-tagged SirR proteins. Lane M: (A, B) protein markers (Fermentas PageRulerTM Unstained Protein Ladder, (C) Sigma-Aldrich bovine albumin marker, (D) In-vitrogen BenchMarkTM Prestained Protein Ladder; lane 1a, b: crude lysate of transformed *E. coli*; lane 2a, b: flow-through fraction from the affinity column; lane 3a, b: wash fraction from the affinity column; lane 4a, b: elution fraction from the affinity column; lane 5a, b: purified recombinant SirR; lane 6: supernatant of His-tagged SirR-expressing *E. coli*; lane 7: pellet of His-tagged SirR-expressing *E. coli*; lane 8: supernatant of His/thioredoxin-tagged SirR-expressing *E. coli*; lane 9: pellet of His/thioredoxin-tagged SirR-expressing *E. coli*.

DISCUSSION

MTB *sirR* has previously been suggested to encode a 25 kDa iron-dependent regulator (Saha *et al*, 2009). Our annotation of the MTB genome confirmed the existence of the *Rv2787-sirR* operon (Cole *et al*, 1998), and showed that the *Rv2787-sirR* operon is transcribed in the opposite direction relative to adjacent genes, with

FadE21 located upstream. MTB *sirR* was co-transcribed with *Rv2787*. TSS was located between -70 bp and -275 bp upstream of the *Rv2787-sirR* operon start codon; bioinformatics analysis suggested that the TSS is located -90 bp from *Rv2787* start codon.

Heterologously expressed His- and His/thioredoxin-tagged MTB SirR pro-

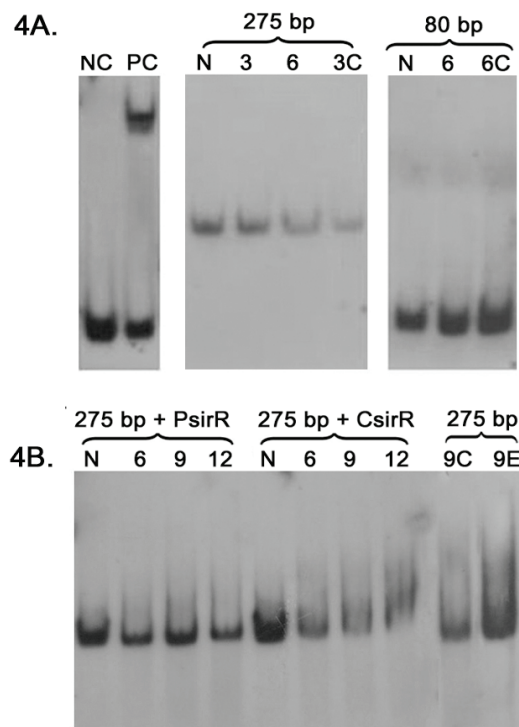


Fig 4—Electrophoretic mobility shift assay of recombinant MTB His-tagged SirR. The experimental protocols are described in Materials and Methods. Lane NC: negative control from the kit; lane PC: positive control from the kit; lane N: reaction without His-tagged SirR; lanes 6, 9 and 12: reactions containing 6, 9 and 12 μ g, respectively of His-tagged SirR or unpurified His-tagged SirR; lane 9C: reaction with 9 μ g of His-tagged SirR in the presence of unlabeled amplicon; lane 9E: reaction with 9 μ g of untransformed *E. coli* protein lysate. 275 bp + P SirR: reaction using labeled 265-bp probe and purified His-tagged SirR; 275 bp + C SirR: reaction using labeled 265-bp probe and unpurified His-tagged SirR; 275 bp: reaction using labeled 275-bp probe.

teins have a molecular weight of 25 kDa (by SDS-PAGE) and exists in solution as a dimer (native PAGE). The crystallographic structure of MTB SirR suggested that two to four molecules assembled in

an asymmetric unit (Saha *et al*, 2009). The addition of a thioredoxin tag was originally performed to increase the solubility of the expressed SirR (LaVallie *et al*, 2000), and that the N-tagged thioredoxin recombinant MTB SirR also exists as a dimer, suggests that the region involved in dimerization is not located at the N-terminus.

SirR has been proposed to be an iron-responsive regulator, but because it contains a putative DNA binding domain as well, we hypothesized that MTB SirR could regulate the expression of its upstream gene, *Rv2787*, a putative chromosome partitioning protein (Camus *et al*, 2002). The DNA binding site of MTB SirR has been predicted by bioinformatics analysis using the binding sequence from *S. epidermidis* (Hill *et al*, 1998) to be located 90 bp upstream of *sirR*. Interestingly, EMSA showed that SirR binds to a cis element located between -1 and -275 bp upstream of the start codon of *Rv2787-sirR* operon. However, EMSA showed a more marked gel shift in the presence of crude SirR preparation, but the loss in probe intensity and smearing of the bands suggest that the phenomenon is probably an artifact.

It has been reported that transcriptional repressors can regulate the expression of their own operons. For example, the TetR family member VarR (*Streptomyces virginiae*) regulates an upstream gene in its own operon (Namwat *et al*, 2001). Self-regulatory systems have been also observed in the TetR family repressor and in the *FurA* operon in MTB (Zahrt *et al*, 2001). Binding of Rv2358 to a region upstream of the *FurB-Rv2358* operon in MTB has been reported (Milano *et al*, 2004).

However, expression of the *Rv2787-sirR* operon may be regulated by other factors. In fact, in the present study, only

slight changes in EMSA were observed after the binding of MTB SirR to its putative cis DNA binding site. There are several factors that could affect the SirR-DNA interaction, such as low binding affinity of the recombinant protein compared with the native form and the requirement for a specific ligand that may not be iron. Previously, a mutation in the iron-responsive repressor IdeR (D117K) could not completely abrogate the transcription of IdeR-regulated genes in the absence of iron, suggesting that other iron-dependent factors regulate iron homeostasis in MTB (Manabe *et al*, 2005).

The limitations of this study were the inability to prove the iron binding property of MTB SirR and to determine the effect of iron on the expression of this gene. Because Rv2787 has been suggested to function in chromosomal partitioning (unpublished data), this role could be further analyzed in the future. Furthermore, construction of isogenic mutants of MTB *sirR* in order to compare the phenotypes with wild type and *sirR* knockout strains, as well as experiments testing the effect of iron on the expression of *sirR* and Rv2787 should be performed to elucidate the exact function of MTB *sirR*.

In summary, the Rv2787-*sirR* operon of MTB was shown to be transcribable. Heterologous expression and purification of recombinant MTB SirR resulted in a protein with properties in agreement with those predicted by bioinformatics analysis. MTB SirR was able to bind to a cis element upstream of the start codon of Rv2787-*sirR* operon, suggesting that it may have a gene regulatory function. As Rv2787-*sirR* operon is uniquely present in MTB and *M. bovis* but not in other mycobacterial species (unpublished data), proteins encoded by this operon may be good targets for development of new anti-TB drugs.

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