

Rv2358 and FurB: Two Transcriptional Regulators from *Mycobacterium tuberculosis* Which Respond to Zinc

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In a previous work, we demonstrated that the *Mycobacterium tuberculosis* Rv2358-*furB* operon is induced by zinc. In this study, the orthologous genes from *Mycobacterium smegmatis* mc²155 were inactivated and mutants analyzed. Rv2358 protein was purified and found to bind upstream of the Rv2358 gene. Binding was inhibited by Zn²⁺ ions.

The long-recognized phenomenon of nutritional immunity, in which the sequestration of iron and possibly other metals occurs as a nonspecific host response to infection (1), hints in general terms at the possibility of a keen competition between host and parasite for essential metal ions. Two major mechanisms play the prominent roles in governing metal ion homeostasis and resistance. The first one involves the uptake or efflux of specific heavy metal ions across biomembranes (15), while the second involves the specific chelation of metals by intracellular chaperons, e.g., metallothioneins (12). In prokaryotes, the expression of these genes is tightly controlled by specific “metal-sensing” transcriptional regulators (3, 5) clustered in four distinct families: Fur (4), DtxR (10), MerR (2), and SmtB/ArsR (3). In each family, representative members are dimers, with each subunit containing an amino-terminal helix-turn-helix DNA-binding domain and an adjacent metal-binding domain.

Mycobacterium tuberculosis genome encodes two regulators of the Fur family (FurA and FurB), two regulators of the DtxR family (IdeR and SirR), three regulators of the MerR family (Rv1674c, Rv1994c, and Rv3334), and seven regulators of the SmtB/ArsR family (Rv0324, Rv0576, Rv2034, Rv2358, Rv2640c, Rv2642, and Rv3744) (<http://genolist.pasteur.fr/TubercuList/>). IdeR is the only one whose role has been well characterized. Like Fur and DtxR, IdeR binds iron and then interacts with a specific sequence in the operator region of iron-regulated genes to control their transcription (11, 13).

The genetic linkage of *furA* and *katG* in all species of mycobacteria has suggested that FurA may control *katG* expression. Indeed, recent studies have demonstrated that it is a negative regulator of *katG* (18). Further, we showed that transcription of both genes is induced upon oxidative stress and that FurA negatively controls transcription of its own gene (7, 14).

In mycobacteria, the *furB* gene is located immediately downstream of the Rv2358 gene, encoding a putative regulator of

the SmtB/ArsR family. Using *Mycobacterium smegmatis* as a model system, we found that the *M. tuberculosis* Rv2358 and *furB* genes are cotranscribed by a common promoter which is induced by zinc (8), thus suggesting that this operon is involved in zinc homeostasis. In this study, we report results on inactivation of *Ms2358* (the Rv2358 ortholog) and *furB* genes in *M. smegmatis* and the in vitro interaction between the Rv2358 protein and a palindromic sequence located immediately upstream of the transcriptional start site of the Rv2358-*furB* operon.

***Ms2358* and *furB* knockout strains and zinc tolerance.** To investigate the physiological role of these genes, we produced two *M. smegmatis* mutants in which *Ms2358* and *furB* were inactivated by partial gene deletion and insertion of a hygromycin resistance cassette, according to the protocol described by Jacobs et al. (6), and the two strains were named mcJA8 and mcJF3, respectively.

In order to confirm the involvement of *Ms2358* and *FurB* in metal homeostasis, we tested the relative resistance of the wild type (wt) and the two mutants to ZnSO₄ and to the chelating agent EDTA by using an agar disk diffusion assay. Filter paper disks soaked with 10 μl of 100 mM ZnSO₄ or 0.5 M EDTA were put onto 20-ml 7H11 plates that were inoculated with 0.5 ml of *M. smegmatis* cultures, grown in 7H9 medium up to an optical density at 600 nm of 0.6. Plates were incubated at 37°C for 48 h and inhibitory boundary measured. While no difference in zinc tolerance could be detected among wild-type and mutant strains, tolerance to EDTA was decreased in mcJA8 but not in mcJF3. The increased sensitivity to chelating agents, shown by the *Ms2358* knockout strain, was partially reversed after complementation with pMVA2, a pMV261 (16) derivative containing a wt copy of *Ms2358* under the control of the *hsp60* promoter (Table 1).

The regulation of the Rv2358-*furB* operon is mediated by Rv2358. To verify whether zinc-dependent expression of the Rv2358-*furB* operon is mediated by Rv2358 or FurB, we analyzed its promoter activity in the wt and in the mutant strains. *M. smegmatis* mc²155, mcJA8, and mcJF3 were transformed with pBT9 and pBT5. These plasmids are pSM128 derivatives carrying different transcriptional fusions of *M. tuberculosis* Rv2358-*furB* promoter region to a promoterless *lacZ* gene (Fig.

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TABLE 1. Sensitivities of *M. smegmatis* strains to EDTA

<i>M. smegmatis</i> strain	Diameter (cm) ^a
mc ² 155	2.2 ± 0.1
mcJF3	2.1 ± 0.1
mcJA8	3.3 ± 0.1
mcJA8(pMVA2)	2.6 ± 0.1

^a The values represent the averages ± the standard deviations of the diameters of the inhibition zones in cm. The experiments, performed in triplicate, were repeated two times with independent bacterial cultures.

1A) (8): pBT9 carries the promoter region of the operon plus a partial *Rv2358* sequence, while pBT5 contains the promoter region and the complete *Rv2358* sequence. After growth under conditions in which metal ions were limited, zinc was added to the cultures at physiological noninducing (1.4 nM) or inducing (100 μM) concentrations, as previously described (8). Cells were disrupted by sonication, and levels of β-galactosidase activity of the extracts were measured as described by Miller (9). The enzyme activity was expressed as nanomoles of *o*-nitrophenol-β-D-galactopyranoside converted to *o*-nitrophenol min⁻¹ mg⁻¹ of total proteins. β-Galactosidase activity of strains transformed with pBT9 revealed that the zinc-dependent regulation of the *Rv2358-furB* promoter was lost in mcJA8 but maintained in mcJF3 (Fig. 1B). Promoter activity was restored in mcJA8 mutant transformed with pBT5 plasmid, thus suggesting a direct involvement of *Rv2358* protein in the transcriptional regulation of the operon.

Rv2358 binds specifically to the *Rv2358-furB* promoter region. The *Rv2358* coding region was cloned into pET-15b (Invitrogen), and the recombinant protein was overproduced in *Escherichia coli* BL21(DE3)plysE (17) and purified. *M. tuberculosis* FurB protein was overproduced with the pGEX-6P-I

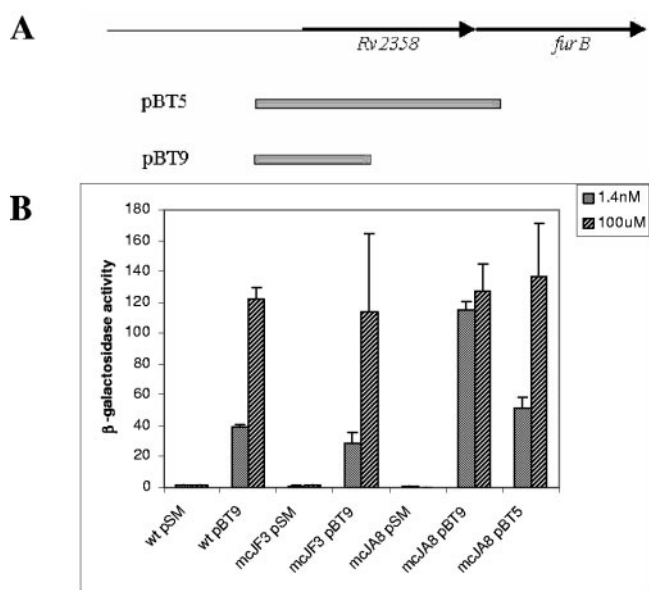


FIG. 1. β-Galactosidase assays of *M. smegmatis* mc²155 wild-type and mcJA8 and mcJF3 mutant strains, assayed at physiological (1.4 nM) and inducing (100 μM) zinc concentrations. (A) pSM derivatives pBT5 and pBT9 containing the promoter fragments of the *Rv2358-furB* operon. (B) β-Galactosidase activity results.

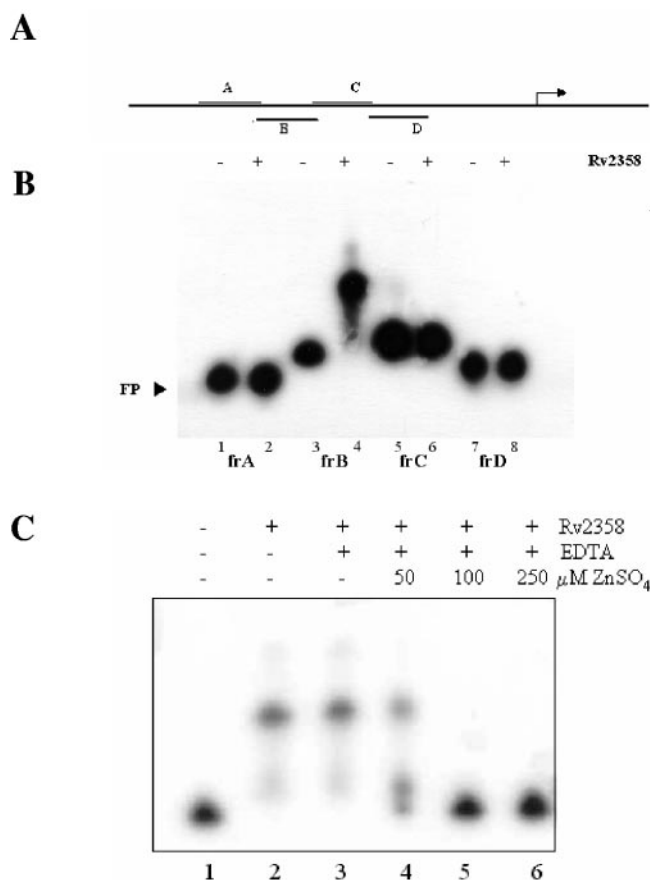


FIG. 2. EMSA experiments and probes used. (A) Fragments A, B, C, and D, mapping in the *Rv2358* promoter region, are shown. (B) EMSA results with fragment A (lanes 1 and 2), fragment B (lanes 3 and 4), fragment C (lanes 5 and 6), and fragment D (lanes 7 and 8) as probes. Odd lanes represent negative controls without *Rv2358* protein. (C) *Rv2358* binding on fragment B is specifically inhibited by the addition of zinc.

system (Pharmacia-Amersham) in *E. coli* XL1-Blue and purified as previously described (8).

Rv2358 was used in electrophoretic mobility shift assays (EMSA) to determine its ability to bind the *Rv2358-furB* promoter region. Overlapping fragments of about 100 bp, mapping on the promoter region contained in pBT9, were amplified by PCR with specific primers from *M. tuberculosis* genomic DNA, end labeled with [γ -³²P]dATP (6,000 Ci/mmol) by using T4 kinase, and used as probes (fragments A, B, C, and D [Fig. 2A]). Binding reaction mixtures [20 mM Tris-HCl, pH 7.8, 1 mM EDTA, 0.1 μg of poly(dI-dC)/ml, 0.5 mM spermidine, 3% glycerol] containing 0.27 pmol of the labeled probe were incubated with purified *Rv2358* protein (5.5 pmol) for 20 min at room temperature. Reaction mixtures were loaded onto a non-denaturing 8% polyacrylamide gel containing 40 mM Tris-acetate, pH 8, and 1 mM EDTA.

Rv2358 was able to retard specifically the migration of fragment B (Fig. 2B, lanes 3 and 4). The specificity of binding was further investigated in competition experiments: gel shift was partially inhibited by a 200-fold excess of unlabeled fragment B

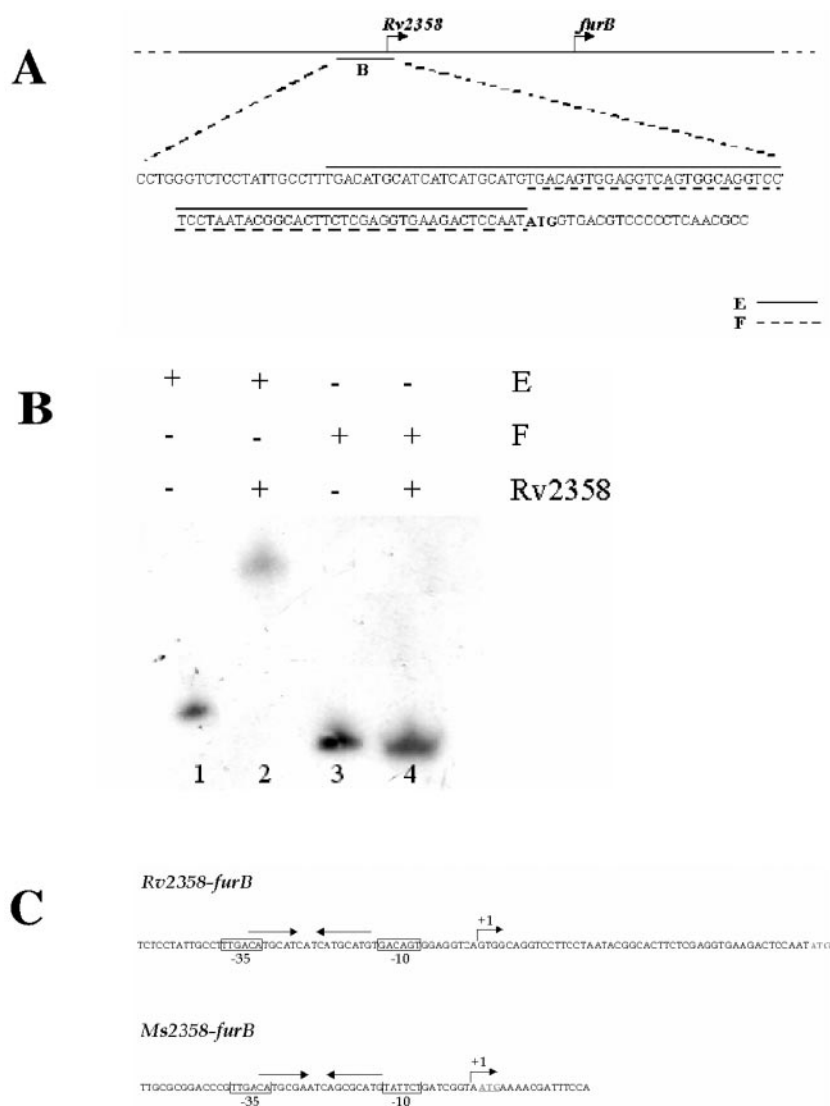


FIG. 3. Identification of the Rv2358 binding site. Fragments E and F, internal to fragment B (A), were used in EMSA experiments, but only fragment E was retained by Rv2358 protein (B). (C) Promoter/operator sequence organization in *M. tuberculosis* and *M. smegmatis*. Palindrome localization between -35 and -10 sites is significantly conserved in *M. tuberculosis* and *M. smegmatis*. The consensus promoter is boxed; transcriptional start point, as mapped by experiments with rapid amplification of cDNA ends, is shown by the curved arrow, and palindrome orientation is shown by straight arrows.

but not by a similar amount of an unrelated DNA fragment (data not shown).

As shown in Fig. 2C, Zn^{2+} inhibits the Rv2358-fragment B complex formation. The shift bands, corresponding to the complex, partially disappeared when the Zn^{2+} concentration was $50 \mu\text{M}$ and completely disappeared when the concentrations of Zn^{2+} were raised to 100 and to $250 \mu\text{M}$, respectively.

In similar experiments, we showed that other divalent metal ions, such as Cd^{2+} , Cu^{2+} , Fe^{2+} , Ni^{2+} , Mn^{2+} , and Ni^{2+} , are unable to inhibit Rv2358-fragment B complex formation (data not shown). Taken together, these findings demonstrate that Rv2358 DNA binding activity is exclusively regulated by Zn^{2+} .

Two different Rv2358-DNA complexes could be detected at intermediate protein concentrations, but only the fast-migrating one was stable when protein-DNA stoichiometry was in-

creased up to 200-fold. The presence of a reducing agent, such as dithiothreitol, also caused the loss of the slower-migrating band (data not shown). These evidences would suggest the labile formation of higher-molecular-weight complexes because of multimerization of Rv2358 on the DNA probe, the stability of which is clearly dependent on the redox state of the protein.

Binding experiments performed under the same conditions with the purified FurB protein were unsuccessful (data not shown), confirming the exclusive role of Rv2358 in the operon regulation.

In order to identify more precisely the Rv2358 binding site, fragment B (126 bp) was divided in two smaller fragments, E and F (83 and 62 bp, respectively) (Fig. 3A). When these two fragments were used in EMSA, only fragment E was found to

be still retained by Rv2358 (Fig. 3B). Sequence analysis of this fragment showed the presence of a palindromic sequence, probably the Rv2358 recognition site, with homology to 12-2-12 inverted repeats, described as binding sites of members of ArsR/SmtB family regulators (3).

Experiments with rapid amplification of cDNA ends mapped the 5' end of *M. tuberculosis* Rv2358-*furB* transcript 12 bp downstream of the previously identified inverted repeats which overlaps the putative -35 (TTGACA) and -10 (GAC AGT) promoter region. Interestingly, we found a similar palindromic sequence in the *Ms2358-furB* upstream region in *M. smegmatis* (Fig. 3C).

Conclusions. From these results, it appears that the Rv(Ms)2358 protein is responsible for the zinc-dependent repression of the *Rv(Ms)2358-furB* operon. This finding is consistent with a simple model of derepression, in which metal binding by the sensor protein weakens the DNA binding affinity significantly, such that RNA polymerase can load and initiate transcription of the operon.

Until now, no regulatory function could be attributed to FurB protein; mycobacterial *furB* could be the first member of the *fur* family not to be autoregulated. Our hypothesis is that Rv2358 could act as the "metal sensor" which controls the expression of FurB in response to zinc availability. On the other hand, FurB could be involved in the regulation of still-undefined metal transport systems. However, we cannot rule out a further role of Rv2358 in zinc homeostasis. Usually, SmtB/ArsR regulators are involved in control of genes responsible for metal-specific efflux pumps, membrane-bound transporters, metal reductases, or metal-sequestering proteins (3). Its zinc-dependent DNA binding activity is in agreement with its hypothetical role in regulation of efflux systems or other resistance determinants, able to remove metal ions at high toxic concentrations.

Our results are in favor of the hypothesis that cotranscription of *Rv(Ms)2358* and *furB* genes could represent the key element for the compensation between import and export of zinc.

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REFERENCES

1. Beisel, W. R. 1977. Magnitude of the host nutritional responses to infection. *Am. J. Clin. Nutr.* **28**:1236-1247.
2. Brown, N. L., J. V. Stoyanov, S. P. Kidd, and J. L. Hobman. 2003. The MerR family of transcriptional regulators. *FEMS Microbiol. Rev.* **27**:145-163.
3. Busenlehner, L. S., M. A. Pennella, and D. P. Giedroc. 2003. The SmtB/ArsR family of metalloregulatory transcriptional repressors: structural insights into prokaryotic metal resistance. *FEMS Microbiol. Rev.* **27**:131-143.
4. Escobar, L., J. Perez-Martin, and V. De Lorenzo. 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. *J. Bacteriol.* **181**:6223-6229.
5. Finney, L. A., and T. V. O'Halloran. 2003. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. *Science* **300**:931-936.
6. Jacobs, W. R., Jr., S. B. Snapper, M. Tuckman, and B. R. Bloom. 1989. Mycobacteriophage vector systems. *Rev. Infect. Dis.* **11**:S404-S410.
7. Milano, A., F. Forti, C. Sala, G. Riccardi, and D. Ghisotti. 2001. Transcriptional regulation of *furA* and *katG* upon oxidative stress in *Mycobacterium smegmatis*. *J. Bacteriol.* **183**:6801-6806.
8. Milano, A., M. Branzoni, F. Canneva, A. Profumo, and G. Riccardi. 2004. The *Mycobacterium tuberculosis* Rv2358-*furB* is a zinc-induced operon. *Res. Microbiol.* **155**:192-200.
9. Miller, J. M. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
10. Oram, D. M., A. Avdalovic, and R. K. Holmes. 2004. Analysis of genes that encode DtxR-like transcriptional regulators in pathogenic and saprophytic corynebacterial species. *Infect. Immun.* **72**:1885-1895.
11. Pohl, E., R. K. Holmes, and W. G. J. Hol. 1999. Crystal structure of the iron-dependent regulator (IdeR) from *Mycobacterium tuberculosis* shows both metal binding site fully occupied. *J. Mol. Biol.* **285**:1145-1156.
12. Robinson, N. J., S. K. Whitehall, and J. S. Cavet. 2001. Microbial metallo-thioneins. *Adv. Microb. Physiol.* **44**:183-213.
13. Rodriguez, G. M., M. I. Voskuil, B. Gold, G. K. Schoolnik, and I. Smith. 2002. *ideR*, an essential gene in *Mycobacterium tuberculosis*: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. *Infect. Immun.* **70**:3371-3381.
14. Sala, C., F. Forti, E. Di Florio, F. Canneva, A. Milano, G. Riccardi, and D. Ghisotti. 2003. *Mycobacterium tuberculosis* FurA autoregulates its own expression. *J. Bacteriol.* **185**:5357-5362.
15. Solioz, M., and C. Vulpe. 1996. CPx-type ATPases: a class of P-type ATPases that pump heavy metals. *Trends Biochem. Sci.* **21**:237-241.
16. Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennet, G. P. Bansall, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom. 1991. New use of BCG for recombinant vaccines. *Nature* **251**:456-460.
17. Studier, Y. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**:60-89.
18. Zahrt, T. C., J. Song, J. Siple, and V. Deretic. 2001. Mycobacterial FurA is a negative regulator of catalase-peroxidase gene *katG*. *Mol. Microbiol.* **39**:1174-1185.



AUTHOR'S CORRECTION

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1a. **Agranoff, D. D., and S. Krishna.** 1998. Metal ion homeostasis and intracellular parasitism. *Mol. Microbiol.* **28**:403–412.