

Identification and characterization of two divergently transcribed iron regulated genes in *Mycobacterium tuberculosis*

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Summary *Setting:* Low iron availability in the host induces the expression of iron acquisition systems and virulence genes in many pathogens. IdeR is a mycobacterial iron dependent regulator that controls the iron starvation and oxidative stress responses in *Mycobacterium smegmatis*. It is important to determine the role of IdeR and its regulon in *M. tuberculosis*, as identification of iron regulated genes can aid in the design of new drugs and generation of attenuated strains.

Objective: A potential IdeR binding site was found in the *M. tuberculosis* genome flanked by two divergently oriented open reading frames, *irg1* and *irg2*. The aim of this study was to determine whether *irg1* and *irg2* were iron and IdeR regulated genes.

Design: Interaction of IdeR with the putative binding sequence was examined by gel shift and footprinting assays. Transcriptional fusions of *irg1* and *irg2* to *lacZ* were used to study the effect of iron levels on the expression of these genes.

Results: IdeR binds to the predicted binding site, which overlaps with the *irg1* promoter. *irg1* and *irg2* expression was decreased by iron in *M. tuberculosis* and in wild type *M. smegmatis*, but not in a *M. smegmatis ideR* mutant.

Conclusion: Two *M. tuberculosis* iron/IdeR regulated genes were identified. *irg1* is predicted to be the *M. tuberculosis hisE* gene, which is involved in histidine biosynthesis. It is directly upstream of the *M. tuberculosis hisG*. *irg2* encodes a putative membrane protein that is a member of the PPE family. © 1999 Harcourt Publishers Ltd

INTRODUCTION

Iron is an essential element for most living organisms. It functions as a catalyst for electron transport and is an essential component of several important enzymes. The human host withholds iron as a defense mechanism against bacterial infections.¹ Like most bacteria, *Mycobacterium tuberculosis*, the causative agent of tuberculosis, should be able to acquire iron in the host in order to survive. In vitro, mycobacteria respond to iron starvation by inducing the synthesis and secretion of siderophores that solubilize iron and are transported across the cell envelope via specific receptors. Two types of secreted

mycobacterial siderophores have been described, exochelins and carboxymycobactins, which are produced by fast growing mycobacteria and members of the *M. tuberculosis* complex, respectively.² In addition, most mycobacteria produce cell-wall associated siderophores called mycobactins. Carboxymycobactins and mycobactins from *M. tuberculosis* share a common core structure, which is derived from salicylate by the addition of serine (or threonine), two lysines and various fatty acids.^{3,4} It has been observed that purified desferricarboxymycobactin can remove iron from transferrin and lactoferritin and transfer it to mycobactin.⁵ However, the role of siderophores in *M. tuberculosis* growing in the host remains unknown. It is not unreasonable to predict that the ability of *M. tuberculosis* to acquire iron is essential for extracellular multiplication in lung cavities. Inside the phagosome in mononuclear phagocytes, *M. tuberculosis* might obtain

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iron released from transferrin, which has been shown to be accessible to this compartment.⁶ However, dissociation of iron from transferrin is likely decreased if acidification is prevented as is the case in the *M. tuberculosis* phagosome.⁷ Under these conditions, synthesis of siderophores might be important for *M. tuberculosis* iron acquisition, but this has not been shown directly.

Although the acquisition of iron is of utmost importance for all living organisms, an excess of this metal can also be detrimental. In aerobic and facultative anaerobic organisms, iron can catalyze the production of toxic oxygen radicals.⁸ Thus, intracellular iron levels are strictly controlled and iron homeostasis is the result of coordinated assimilation, utilization and storage of iron. In many gram-negative and a few gram-positive bacteria, biosynthesis of siderophores and their receptors is controlled by the ferric uptake regulator Fur. In a complex with iron, Fur binds to a consensus nucleotide sequence in the operator region of Fur-regulated genes and represses their transcription.⁹ In addition to genes involved in siderophore synthesis, a number of other genes not directly related to iron acquisition are included within the Fur regulon. Fur appears to be a pleiotropic regulator controlling diverse processes such as iron metabolism, response to oxidative stress, the acid tolerance response, sugar metabolism and toxin synthesis.¹⁰ DtxR (diphtheria toxin regulator) is a functional homolog of Fur initially discovered in *Corynebacterium diphtheriae* where it regulates both toxin and siderophore production.¹¹ Homologs of DtxR have been identified in *Brevibacterium lactofermentum*¹⁴ and *Streptomyces pilosus*,¹⁵ as well as mycobacteria, where this protein has been named IdeR (iron dependent regulator).^{12,13} IdeR is present in all mycobacteria examined, including virulent and non-virulent species.¹² Inactivation of the *ideR* gene in *M. smegmatis* demonstrated that IdeR negatively regulates siderophore production under iron sufficient conditions.¹⁶ Direct evidence for this role has been obtained as IdeR binds to and regulates the *M. smegmatis* exochelin biosynthetic gene *fxbA* which contains a DtxR/IdeR binding site (iron box) in its promoter.^{17,18} In addition, a *M. smegmatis ideR* mutant is affected in its ability to respond to oxidative stress. This defect is probably a result of the decreased levels of manganese superoxide dismutase and catalase/peroxidase (KatG) activities observed in this mutant.^{16,19}

The nature of the processes known to be controlled by IdeR in *M. smegmatis*, i.e. iron uptake and oxidative stress response, underline the importance of studying the role of this protein and its target genes in *M. tuberculosis*. In this study we begin the identification of *M. tuberculosis* genes that are regulated by IdeR. We characterize a functional IdeR binding site shared by two genes that are divergently transcribed. Both genes were found to be repressed by iron only in the presence of IdeR.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* was routinely grown in Luria-Bertani (LB) medium (Difco). Mycobacterial strains were grown in Middlebrook 7H10 solid medium (Difco) supplemented with 0.2% glycerol and 0.05% Tween 80, and in the case of *M. tuberculosis* also added were 0.5% bovine serum albumin fraction V, 0.2% dextrose and 0.085% NaCl (ADN supplement). When required, streptomycin was added at 20 µg/ml. For detection of β-galactosidase activity, *M. smegmatis* strains were grown in the minimal medium described previously,²⁰ plus 0.05% Tween-80 (MMT). *M. tuberculosis* was grown in MMT plus ADN supplement (MMT-ADN). These media were made low in iron by overnight treatment with Chelex (Chelex 100, BioRad) according to the manufacturer's instructions. Chelex treated medium contained less than 1 µM residual iron as determined by atomic absorption spectroscopy. For growth in high iron, MMT and MMT-ADN were supplemented with 50 µM FeCl₃. *M. smegmatis* mc²155²¹ and *M. tuberculosis* H37Rv were made competent and transformed by electroporation.²²

DNA techniques

DNA manipulations were performed by standard procedures.²³ Restriction and modifying enzymes were obtained from Promega. DNA fragments used in the cloning procedures and PCR products were isolated from agarose gels with the Qiaex or Qiaquick gel extraction Kits (Qiagen Inc.) according to the manufacturer's instructions.

Gel retardation assay

The gel retardation assay was essentially the same as that described by Hamoen et al.,²⁴ with minor modifications. The IdeR protein was purified by nickel sepharose chromatography as previously described.¹³ A 161 bp PCR fragment containing the putative DtxR consensus binding site of the *irg1/irg2* intergenic region was PCR amplified from the *M. tuberculosis* H37Rv genome using the oligonucleotides PE.2 (5'GATTGTTGCACGTGTCAGCC3') and Ppe.2 (5'CTGCCAACCACATCGGGAAGGTCAC3').

The amplified DNA was end labeled with T4 polynucleotide kinase and [³²P]ATP (NEN). Binding reactions, in a buffer composed of 20 mM Tris-HCl, pH 8, 50 mM KCl, 5 mM MgCl₂, 50 µg/ml poly[d(I-C)], 50 µg/ml BSA and 10% glycerol, containing approximately 0.1 picomoles of the radiolabelled *irg1/irg2* DNA fragment (20 000 CPM) were incubated with purified IdeR in 20 µl volumes for 30 min at room temperature. 200 µM NiSO₄ was added to binding reactions and when required, to the acrylamide

Table 1 Bacterial strains and plasmids used in this work

Strain/plasmid	Characteristics	Source/reference
<i>Strain</i>		
<i>E. coli</i> JM109	F' traD36 proA+proB+lacIq lacZΔM15 recA1endA1 gyrA96thi hsdR17 supE44 relA1Δ (lac-proAB) mcrA	Lab strain
<i>M. tuberculosis</i>		
H37Rv		ATCC
ST7	H37Rv with integrated pSM128* (Sm ^R)	This work
ST14	H37Rv with integrated pSM309 (Sm ^R)	This work
ST15	H37Rv with integrated pSM312 (Sm ^R)	This work
ST23	H37Rv with integrated pSM328 (Sm ^R)	This work
<i>M. smegmatis</i>		
mc ² 155	High transformation mutant	(21)
SM17	mc ² 155 with integrated pSM128 (Sm ^R)	(18)
SM115	mc ² 155 with integrated pSM309 (Sm ^R)	This work
SM123	mc ² 155 with integrated pSM312 (Sm ^R)	This work
SM130	mc ² 155 with integrated pSM328 (Sm ^R)	This work
SM3	mc ² 155 with <i>ideR::myd200</i> disruption (Km ^R)	(16)
SM60	SM3 with integrated pSM128 (Km ^R , Sm ^R)	(18)
SM116	SM3 with integrated pSM309 (Km ^R , Sm ^R)	This work
SM124	SM3 with integrated pSM312 (Km ^R , Sm ^R)	This work
SM131	SM3 with integrated pSM328 (Km ^R , Sm ^R)	This work
<i>Plasmid</i>		
pSM128	Integrative* shuttle promoter-probe vector for mycobacteria (Sm ^R)	(18)
pSM236	pUC19 carrying the <i>M. tuberculosis sigA</i> promoter (Amp ^R)	(25)
pSM309	pSM128 carrying the <i>irg1-lacZ</i> fusion (Sm ^R).	This work
pSM312	pSM128 carrying the <i>irg2-lacZ</i> fusion (Sm ^R)	This work
pSM328	pSM128 carrying the <i>M. tuberculosis sigA-lacZ</i> fusion (Sm ^R)	This work
pSM304	pUC19 carrying the upstream region of <i>irg1</i> (Amp ^R)	This work

*pSM128 and its derivatives contain the mycobacteriophage L5 *int* system that promotes integration at the chromosomal L5 *att* site. Km^R, kanamycin resistant; Sm^R, streptomycin resistant; Amp^R, ampicillin resistant.

gels and running buffers. Of each reaction, 15 µl were loaded without dye to a 8% polyacrylamide gel containing 40 mM Tris-acetate, pH 8. Gels were run at 110 volts at room temperature, dried and radioactivity was visualized by autoradiography. For the competition experiments, cold competitor DNA was mixed with the same ³²P-labeled *irg1/irg2* DNA fragment described earlier in this paragraph, prior to addition of IdeR. To make competitor DNAs, a 280 bp *sigA* fragment beginning 240 bp upstream of the *sigA* translational start codon and 40 bp of the coding sequence was PCR amplified from the *M. tuberculosis* H37Rv chromosome using the primers

OMTA-1 and OMTA-2 (25); a 126 bp *fxbA* fragment containing the promoter and IdeR binding site was PCR amplified from the *M. smegmatis* chromosome using the primers FXB5'2 (5'-GTGGTGGTCTTCCCCCTGGC-3') and FXB3'7 (5'-TGGCAGGTTCCGGGGCGG-3').¹⁸ In the competition experiments, a gel shift was performed with ³²P-labelled *irg1/irg2* DNA fragment incubated with 4.5 picomoles of IdeR and different amounts of competing DNA. This amount of protein gives a partial gel shift, indicating that IdeR is limiting, a condition required for the competition experiments. Concentrations of competitor DNA were progressively increased in 2-fold increments

from 0–5 pmoles, calculated on the basis of DNA fragment (not nucleotide) concentration. The radioactivity in the shifted bands in the B and D gels was quantitated by densitometric scanning.

DNase footprint analysis

Oligonucleotides PE.2 and Ppe.2, described above, corresponding to both strands of the *irg1/irg2* promoter region were labeled with T4 polynucleotide kinase and [$\gamma^{32}\text{P}$] ATP and then used to individually PCR amplify the 161 bp fragment containing the IdeR binding site in the presence of the non-labelled cognate oligonucleotide. This resulted in two DNA fragments that were 5' end labelled on each strand. The end labelled DNAs were allowed to bind to IdeR using the same conditions as in the gel retardation experiments. After incubation for 30 min at room temperature reaction volumes were adjusted to 50 μl with water and then 50 μl of a $\text{Ca}^{2+}/\text{Mg}^{2+}$ solution (5 mM CaCl_2 and 10 mM MgCl_2) were added. Then, 0.15U of DNase I was added and mixtures were incubated for 1 min at room temperature. Reactions were terminated by the addition of 90 μl of stop solution (200 mM NaCl, 20 mM EDTA, 1% SDS and 100 $\mu\text{g}/\text{ml}$ yeast RNA). Samples were subsequently extracted with phenol/chloroform, ethanol precipitated and resuspended in a formamide loading dye. Samples were electrophoresed on a 6% TBE polyacrylamide-urea sequencing gel and analyzed by autoradiography. Maxam-Gilbert A + G sequencing reactions were used to locate the protected regions.²⁶

RNA isolation and transcriptional start point mapping

RNA from *M. tuberculosis* H37Rv grown in MMT-ADN low iron was prepared by mechanical disruption of bacteria with glass beads in the presence of phenol and lithium chloride.²⁷ The *irg1* transcriptional start point (TSP) was determined by primer extension analysis, following previously described methods²⁷ with minor modifications. Briefly, the oligonucleotide Ppe.1 (5'CCGAGTTCGGC-GAACAGATCCTC3'), complementary to the sequence between nucleotides 31 to 53 relative to the first nucleotide in the *irg1* start codon, was 5'-end labelled with [$\gamma^{32}\text{P}$] ATP using T4 polynucleotide kinase. 15 picomoles of end labeled Ppe.1 primer were annealed to 60 μg of RNA (1 min at 90°C, 2 min at 60°C, 10 min on ice) and then extended with AMV reverse transcriptase (USB) (15 min at 48°C). To determine the size of the extended product, the plasmid pSM304 and the primer Ppe.1 were used to generate a sequencing ladder by the dideoxy chain-termination method with Sequenase T7 DNA polymerase (Sequenase 2.0, Amersham). Primer extension products were loaded onto a 6% polyacrylamide sequencing gel along with the sequencing ladder.

Construction of lacZ transcriptional fusions

A 180 bp DNA fragment containing the putative IdeR binding site and promoter regions of *irg1* and *irg2* was amplified by PCR from the *M. tuberculosis* H37Rv chromosome using the primers Ppe.1 and Ppe.2, described above. This PCR fragment was cloned in both orientations in the *Scal* site of pSM128 to generate the plasmids pSM309 and pSM312. Plasmid pSM309 contains the *irg1-lacZ* transcriptional fusion and pSM312 contains the *irg2-lacZ* fusion. Plasmid pSM328 carries a *sigA-lacZ* fusion. It was constructed by subcloning the 280 bp fragment from pSM236 that contains the *M. tuberculosis sigA* promoter into the *Scal* site of pSM128.

β -galactosidase assay

Cultures of *M. tuberculosis* or *M. smegmatis* strains harboring the different *lacZ* fusions were grown in the appropriate medium (low or high iron), collected at an OD_{600} of 0.5–0.7, washed and resuspended in Z buffer.²⁸ Suspensions were mixed with zirconia beads and were lysed by three pulses of 30 s in a Mini Bead-beater (Biospec Products). Protein concentration of the extracts was estimated by using the Bio-Rad protein assay (Bio-Rad), with bovine serum albumin as the standard. β -galactosidase activity of the extracts was determined by using Miller's method²⁸ and are expressed as nanomoles of nitrophenol /min/mg protein.

RESULTS

Identification of a putative IdeR binding site in the *M. tuberculosis* chromosome

It has previously been shown that IdeR is a structural and functional homologue of the *C. diphtheriae* DtxR. IdeR binds in vitro to the promoters containing the DtxR binding sequence and complements a *C. diphtheriae* dtxR mutant for iron dependent repression of toxin and siderophore production.¹³ Based on these observations and in order to clone IdeR regulated genes, we used the consensus DtxR binding sequence (iron box) to probe the Sanger *M. tuberculosis* genome database. The consensus DtxR iron box had been determined by sequence analysis of DtxR-dependent operators cloned from genomic libraries of *C. diphtheriae*^{29,30} and by biochemical affinity methods (CAST).³¹ In an initial search of the *M. tuberculosis* H37Rv genome with the DtxR consensus sequence as query, using the BLASTN algorithm with default parameters,³² we identified one putative iron box (IB-1). IB-1 has 16/19 nucleotide matches with the DtxR consensus binding sequence. Visual inspection of DNA sequences adjacent to IB-1 indicated the presence of a second putative iron box (IB-2). IB-2 has 11/19 matches with the DtxR

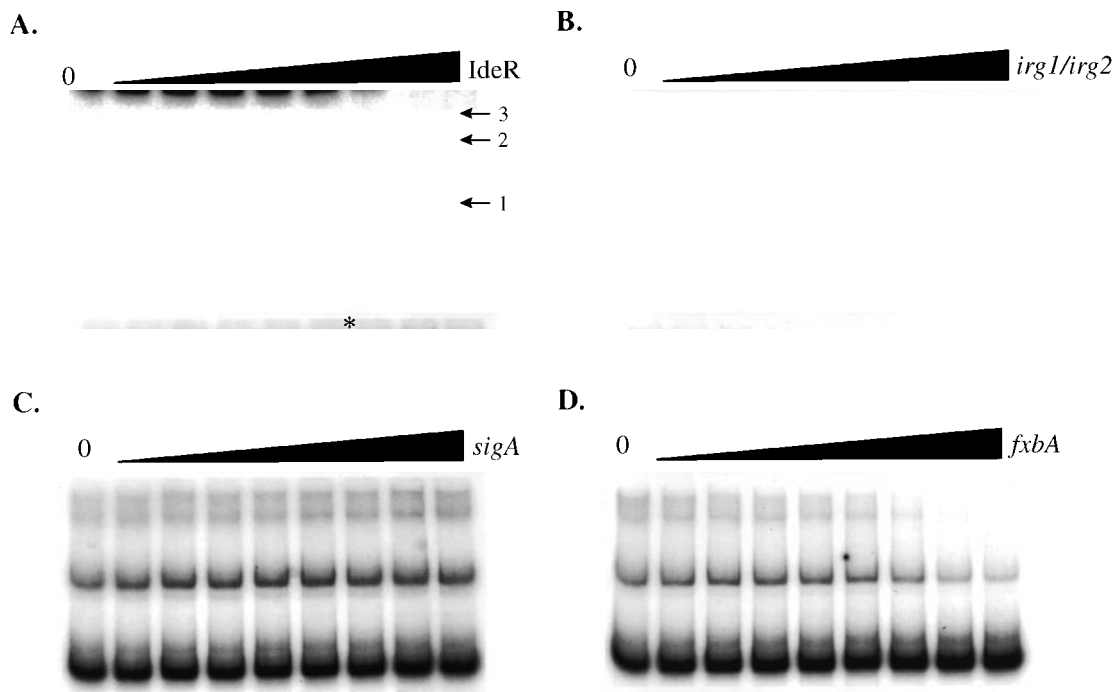


Fig. 2 Gel retardation assays. A 161 bp ^{32}P -labelled fragment, containing the putative IdeR binding site in the *irg1/irg2* intergenic region was incubated with 2-fold increasing concentrations of IdeR (0–18 pmoles) in the presence of Ni^{2+} (A). The arrows and numbers indicate the different retarded DNA species. To test the relative binding affinities of IdeR and the *irg1/irg2* intergenic region with another DNA fragment containing a characterized iron box, competition assays were performed using increasing amounts of cold *irg1/irg2* (B) and the *M. smegmatis fxbA* promoter (D). As a control for non-specific binding by DNA that lacks an IdeR binding site, we also used equivalent amounts of *M. tuberculosis sigA* promoter region (C). In the competition experiments, a similar gel shift as illustrated in (A) was performed with ^{32}P -labelled *irg1/irg2* DNA fragment incubated with 4.5 picomoles of IdeR and different amounts of competing DNA. The asterisk in (A) indicates the unretarded gel shift with this protein concentration. Concentrations of competitor DNA were progressively increased in 2-fold increments from 0 to 5 pmoles. The radioactivity in the shifted bands in the B and D gels was quantitated by densitometric scanning.

We also used competition to assess the relative affinity of IdeR for the *M. smegmatis fxbA* iron box (Figs 2B & D). The iron repressed *fxbA* encodes a formyltransferase involved in exochelin biosynthesis and its promoter has a functional IdeR binding site.^{17,18} Densitometric scanning of the gels in Figure 2B and D was used to quantitate the decrease in radioactivity in the shifted DNA complexes with increasing amounts of both competitor DNAs (data not shown). These measurements indicated that 16 and 12 times the amount of *fxbA* DNA, compared to the *irg1/irg2* fragment, were needed to compete off 50% of the labelled *irg1/irg2* DNA from the larger DNA complexes (2 and 3) and from the faster migrating complex,¹ respectively. To locate the sequences of the *irg1/irg2* intergenic region to which IdeR binds and to ascertain whether both putative iron boxes were protected, we performed DNase I protection assays. In these experiments, using the same 161 bp fragment carrying IB-1 and IB-2 that was used for the gel retardation assays, IdeR was found to protect a region common to the two strands over a 41 bp region including the two predicted iron boxes (Fig. 3).

Transcriptional start point mapping

To identify the TSP of *irg1* and *irg2*, total RNA was isolated from *M. tuberculosis* growing under low iron conditions and primer extension of the transcripts was performed. A 5' terminus of the *irg1* mRNA was identified at position –74 relative to the first nucleotide of the putative *irg1* start codon, using the oligonucleotide Ppe.1 as primer (Fig. 4A). Several other shorter primer extension products were observed, as well. The same products were observed when primer extensions were performed with a second oligonucleotide 20 bp upstream from Ppe.1 (data not shown). We assume that the longest product represents a true TSP, but the significance of the shorter ones, i.e. whether they represent other TSPs or RNA processing/degradation of the longest transcript is not known. Sequences with similarity to the –35 consensus for *E. coli* σ^{70} dependent promoters TTGACA³⁵ and to the –10 region of *M. tuberculosis* promoters TAYGAT (where Y is a pyrimidine base)³⁶ were identified upstream of the longest *irg1* TSP (Fig. 4B). Thus, the IdeR binding site overlaps

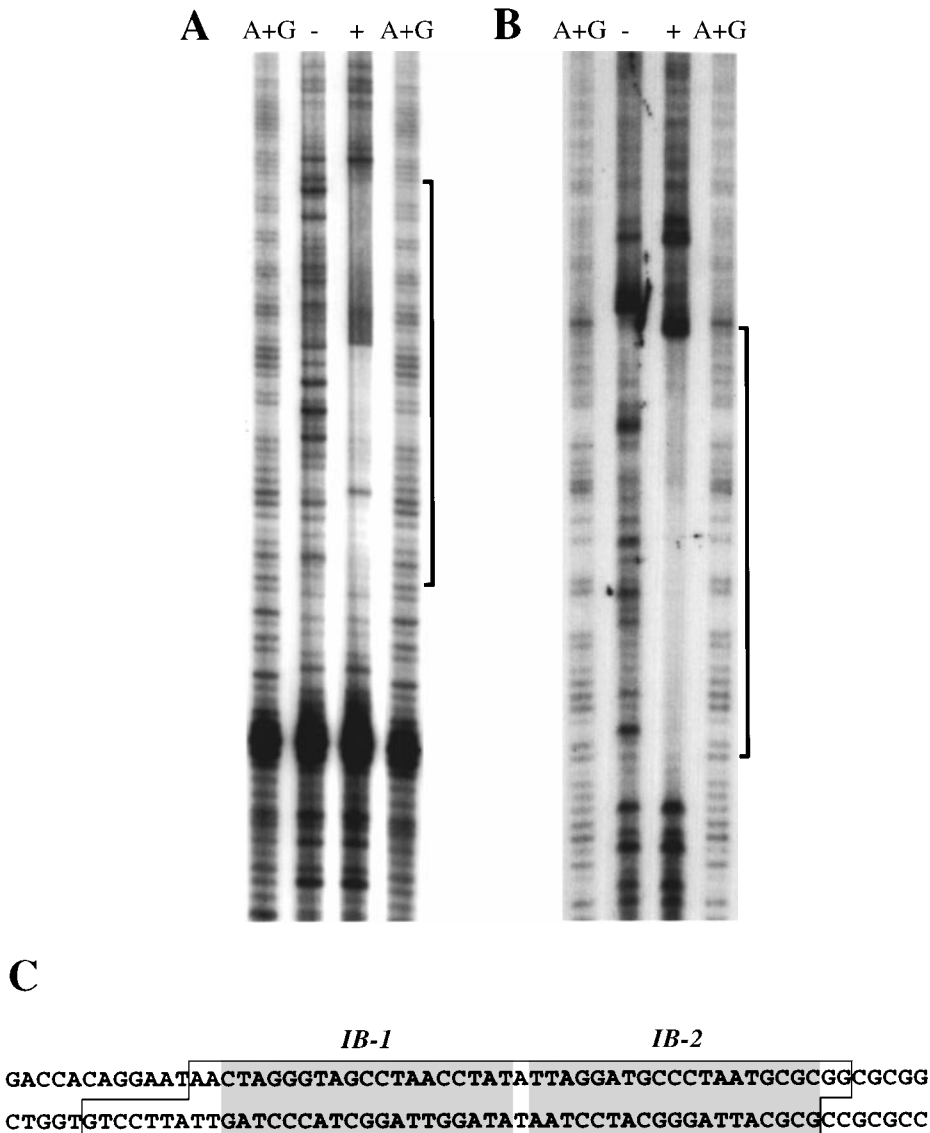


Fig. 3 DNase I protection assay. A 161 bp fragment containing the putative regulatory region of *irg1/irg2*, labeled on the top or coding (A) and the bottom or non-coding strand (B) (as defined by their relation to *irg1*) was incubated with 200 μ M Ni^{2+} with (+) or without (-) 18 picomoles of IdeR. Binding and DNase I digestion conditions are described in Materials and Methods. Maxam and Gilbert A+G sequencing reactions were performed on both strands. Brackets indicate the sequences protected by IdeR from DNase I digestion. The DNA sequence of the *irg1/irg2* regulatory region is shown in C. The boxed region indicates the IdeR binding site, i.e. sequences protected by IdeR from DNase I digestion. The iron boxes are shown in a gray background.

the -10 region and the major TSP, indicating that binding of this protein to this sequence should repress *irg1*. Repeated attempts to determine the TSP of *irg2* by primer extension with the same RNA preparations used for *irg1* were unsuccessful. We believe the amount of *irg2* mRNA may be too low to be detected by primer extension, as suggested by the experiments presented in the next section (Table 2).

Transcriptional fusion studies in *M. tuberculosis*

To determine if *irg2* was transcribed, since we could not detect its mRNA by primer extension and also to determine whether *irg1* and *irg2* expression was regulated by iron, we performed *lacZ* reporter studies with these genes. A DNA fragment containing the two iron boxes and the promoter regions of these genes was cloned into the inte-

Table 2 Expression of *irg1* and *irg2* in *M. tuberculosis* H37Rv and *M. smegmatis* wild type and *ideR* mutant backgrounds

Strain	IdeR	β -galactosidase specific activity ^a	
		Low iron ^b	High iron ^b
<i>M. tuberculosis</i>			
ST7 (pSM128)	+	1±0.3	1±0.3
ST14 (<i>irg1-lacZ</i>)	+	61±8	12±2
ST15 (<i>irg2-lacZ</i>)	+	20±1	6±1
ST23 (<i>sigA-lacZ</i>)	+	120±4	127±5
<i>M. smegmatis</i>			
SM17 (pSM128)	+	4±1	4±1
SM60 (pSM128)	-	5±1	8±2
SM115 (<i>irg1-lacZ</i>)	+	109±10	22±3
SM116 (<i>irg1-lacZ</i>)	-	120±5	113±6
SM123 (<i>irg2-lacZ</i>)	+	41±5	8±1
SM124 (<i>irg2-lacZ</i>)	-	41±4	41±1
SM130 (<i>sigA-lacZ</i>)	+	136±1	135±11
SM131 (<i>sigA-lacZ</i>)	-	180±10	161±6

^ananomoles of nitrophenol/min/mg protein. Values are the mean±standard deviations of three independent experiments. The values obtained with strains carrying pSM128 have not been subtracted from values obtained with the others.

^bLow iron, MMT-ADN medium chelex treated; high iron, MMT-ADN medium chelex treated and supplemented with 50 μ M FeCl₃.

vitro as a binding site for IdeR and also that these genes are transcriptionally regulated by iron in *M. tuberculosis*. To determine if the iron dependent repression of *irg1* and *irg2* is mediated by IdeR, expression of β -galactosidase was determined for each promoter in cultures of the wild type *M. smegmatis* mc²155 and its isogenic *ideR* mutant derivative SM3¹⁶ that had been transformed with the plasmids pSM309, pSM312, pSM328 and pSM128. As observed in *M. tuberculosis*, *irg1* and *irg2* but not *sigA* were repressed by iron in *M. smegmatis* mc²155 (Table 2). The β -galactosidase activity from SM115 and SM123 containing the *irg1* and *irg2 lacZ* fusions was decreased approximately 5-fold when cultured in high iron conditions. However, the β -galactosidase activity from the *ideR* mutant strains SM116 and SM124, containing the *irg1* and *irg2-lacZ* fusions, respectively, remained high, regardless of iron levels (Table 2). Thus, expression of *irg1* and *irg2* is iron independent in the absence of IdeR. These results confirmed that IdeR is required for the iron mediated repression of these genes.

DISCUSSION

We seek to understand the role of IdeR and iron regulated processes in the ability of *M. tuberculosis* to adapt to the environment found in the host and ultimately to cause disease. In this study we have initiated the characterization of the IdeR regulon in *M. tuberculosis*. We identified a sequence from *M. tuberculosis* that contains two adjacent IdeR binding sites and is located in the intergenic region of two divergent open reading frames, *irg1* and *irg2*. Gel

retardation and DNase I protection experiments demonstrated that IdeR binds to a region containing both iron boxes in a metal dependent manner. Tandem arrangement of iron boxes has not been observed in any of the known promoter/operator sequences of DtxR regulated promoters,³⁷ in the IdeR box present in the *M. smegmatis* *fxbA* promoter,¹⁸ nor in the approximately 30 genes we have recently begun to study in *M. tuberculosis* that have potential IdeR binding sites in their promoter regions and are putatively repressed by IdeR (discussed below). Duplication of the iron box could influence the binding affinity of IdeR, and we observed that the *irg1/irg2* fragment bound much more efficiently to IdeR when compared with the *M. smegmatis* *fxbA* promoter region that has a single iron box (Fig. 2). On the other hand this greater affinity could be due to specific sequence elements in one or both of the iron boxes of the *M. tuberculosis* gene and not to the contribution of a second site. As more IdeR regulated mycobacterial promoters are characterized, comparison of IdeR binding affinities to different types of iron boxes can be done as well as identification of key nucleotides within the binding sequences by mutational analyses. Gel retardation studies with IdeR and the *irg1/irg2* region demonstrated the presence of three DNA forms that were retarded by their interaction with IdeR (Fig. 2A). In these studies, the fastest migrating species appeared at the lowest IdeR concentrations and then disappeared at higher protein levels concomitantly with the appearance of the slower migrating forms, suggesting the sequential formation of DNA/protein complexes with one and then additional IdeR dimers. This is not surprising since proteins of the DtxR/IdeR family can form complexes with their operators in forms higher than dimers, i.e. the *C. diphtheriae* *tox* promoter interacts with two dimeric DtxR molecules.³⁸

Determination of the transcriptional start point and promoter sequences of *irg1* indicated that the IdeR binding site overlaps the -10 region of the *irg1* promoter. This finding is consistent with previous observations from DtxR operator sites³⁷ and the *M. smegmatis* *fxbA* promoter^{17,18} where the iron box also overlaps the -10 sequence. Our new observations support the current model in which the binding of DtxR or IdeR to the operator interferes with the binding of the RNA polymerase to the promoter, thereby blocking initiation of transcription.

The IdeR binding site identified in this study is a regulatory element for the transcription of *irg1* and *irg2*. Experiments using transcriptional fusions indicated that the expression of both genes is repressed by iron in *M. tuberculosis*. Although an IdeR mutant in *M. tuberculosis* has not been obtained, we were able to test the requirement of IdeR for the regulation of *irg1* and *irg2* using a *M. smegmatis* *ideR* mutant. As predicted, IdeR is essential for the repression of these two genes in high iron, which

confirmed that *irg1* and *irg2* are iron and IdeR regulated genes. Additionally, the levels of β -galactosidase activity detected in the *ideR* mutant strains containing *irg1* and *irg2-lacZ* reporter fusions grown in high or low iron were similar to those of the equivalent wild-type strains grown in low iron conditions (Table 2). This indicates that IdeR is the sole iron dependent repressor of these genes and also that this protein is not necessary for the expression of *irg1* or *irg2* under non-repressing conditions in the heterologous host *M. smegmatis*. This is unlike the case of the *M. smegmatis fxbA*, whose maximum expression requires the presence of IdeR.¹⁸

The predicted *irg1* gene product shows high similarity to the C-terminal HisE domains of the fused HisE proteins of *S. typhimurium*, *E. coli* and *Synechocystis* and shows little or no similarity to the N-terminal HisI domains of these proteins. Since no other open reading frame similar to HisE was found in the completely sequenced *M. tuberculosis* genome,³⁴ we propose that *irg1* is the mycobacterial *hisE*. The biosynthesis of histidine has been well characterized at the genetic level especially in *S. typhimurium* and *E. coli*.³³ The 11 enzymatic functions required in these enteric bacteria to synthesize histidine are encoded by eight genes, all of which lie in a single operon in the order *his-GDCBHAF(IE)*. In *E. coli* and *S. typhimurium*, *hisLE*, *hisB* and *hisD* encode bifunctional enzymes.³³ The sequence data discussed above indicates that the *M. tuberculosis hisE* encodes a monofunctional enzyme. Unifunctional HisE proteins have also been described in *Streptomyces coelicolor*,³⁹ *Azospirillum brasilense*,⁴⁰ *Rhodobacter sphaeroides* and *Methanococcus vannielii*.⁴¹ In addition, analysis of the *M. tuberculosis* genome sequence reveals that *hisE* is not linked to the rest of the *his* operon, which has the structure *his-DCBHA(impA)FI*.³⁴ The *hisI* gene in this gene cluster (annotated as *hisI2*) is 38% identical and 61% conserved when compared to the N-terminal HisI domains encoded by the fused *S. typhimurium* and *E. coli hisLE* genes and shows little or no similarity to their C-terminal HisE domains. This indicates that the mycobacterial HisI, like HisE, is a monofunctional enzyme. Immediately downstream of the *M. tuberculosis hisE* is the unique *hisG*. HisG and HisE are respectively the first and third enzymes in the histidine biosynthesis pathway.³³ The proximity of *hisE* and *hisG* suggests that they are co-transcribed, but further studies will be required to answer this question. Similarly to what is found in *M. tuberculosis*, the *M. smegmatis hisG* is not found linked to *hisD* and *hisC*.⁴²

Approximately 41 ATP molecules are required to synthesize each histidine molecule.³³ For this reason it is not surprising that microorganisms possess an elaborate network to control the rate of histidine production. In *E. coli*, regulation of histidine gene expression and activity of the biosynthetic enzymes includes various types of

transcriptional control, feedback inhibition and metabolic regulation.³³ Regulation of histidine biosynthesis has not been extensively studied in mycobacteria, but the organization of the *his* genes in *M. Smegmatis* and *M. tuberculosis* suggest that the mycobacterial mechanism differs from the *S. typhimurium* and *E. coli* paradigm. Our findings that high levels of iron repress the transcription of the *M. tuberculosis hisE* and presumably *hisG* suggest that they are regulated independently from the rest of the *his* operon. However, this repression is not total and *M. tuberculosis* does not behave as a histidine auxotroph when growing in high iron (unpublished experiments), suggesting that enough histidine is synthesized in high iron conditions to maintain normal growth. Histidine at concentrations of up to 10 mM did not affect the expression of *hisE* in *M. tuberculosis* growing in a low iron medium, as determined in β -galactosidase reporter experiments with the *irg1-lacZ* fusion (data not shown). To our knowledge this is the first time that regulation by iron of a histidine biosynthesis gene has been observed. These observations raise the question of why histidine biosynthesis would be regulated by iron in *M. tuberculosis*. One possibility is that histidine, a derivative or a precursor in histidine biosynthesis could be part of a yet to be identified element of the iron uptake system in *M. tuberculosis*, even though only one siderophore biosynthetic operon, i.e. that for mycobactin, has been identified in the *M. tuberculosis* genome^{4,34} and histidine or related molecules are not known to be a part of the mycobactin or carboxymycobactin structure.³ There are a few examples of histidine derivatives being incorporated in siderophores: β -hydroxyhistidine is a component of the exochelin from *M. neoaurum*⁴³ and a pyoverdinin-type siderophore from *Pseudomonas fluorescens*.⁴⁴ Decarboxylation of histidine generates histamine which is a precursor in the biosynthesis of anguibactin, a siderophore from *Vibrium anguillarum*.⁴⁵ Alternatively, regulation of histidine biosynthesis by iron might not be directly related with iron acquisition since, as discussed above, Fur is known to regulate processes that have no apparent relation to iron uptake. We are currently investigating the role of the *M. tuberculosis* HisE in iron acquisition and histidine biosynthesis.

Available information for *irg2* is more limited than for *irg1*. *irg2* which contains the PPE domain that places it in the PPE family of *M. tuberculosis* proteins. Although this family has 68 members, no function has been assigned to any of them.³⁴ Since *irg2* has the profile of a putative membrane protein, it is tempting to speculate that it might have a role in siderophore uptake and we are investigating this possibility.

During the completion of the experiments reported here, two studies appeared on the characterization of *M. tuberculosis* proteins whose levels were regulated by different iron concentrations. These experiments, using

one and two dimensional gel electrophoresis of protein extracts of *M. tuberculosis* grown in high and low iron, noted 17 proteins that were present in greater amounts during iron starvation, while 12 others were diminished.^{46,47}

Of these proteins, five that were present in greater amounts in high iron were identified, as well as 5 that had higher levels in low iron. However, the role of IdeR in the regulation of the structural genes involved in this regulatory response has not been described.

Results presented in this report indicate that a relatively simple search of the *M. tuberculosis* genomic data base permitted the identification of two divergently expressed genes that have a functional IdeR binding site in their promoter regions. With the success of this approach as a validation, we have recently started using the GCG program FINDPATTERNS⁴⁸ to query the Sanger and TIGR *M. tuberculosis* genome databases. This search algorithm is much more sophisticated than the BLASTN program we originally used to find *irg1*. The new search has allowed us to identify additional sequences resembling the DtxR consensus binding site directly upstream of approximately 30 genes. Among the genes are some potentially involved in early steps in aromatic amino acid biosynthesis, cell envelope restructuring and iron acquisition/storage. Preliminary experiments with the promoter/operator regions of four of these genes indicate they specifically interact with IdeR and are also regulated by iron (unpublished experiments). All these putative members of the *M. tuberculosis* IdeR regulon are currently being investigated as to their interaction with IdeR and their roles in *M. tuberculosis* physiology and virulence.

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