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ideR, an Essential Gene in *Mycobacterium tuberculosis*: Role of IdeR in Iron-Dependent Gene Expression, Iron Metabolism, and Oxidative Stress Response†

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The mycobacterial IdeR protein is a metal-dependent regulator of the DtxR (diphtheria toxin repressor) family. In the presence of iron, it binds to a specific DNA sequence in the promoter regions of the genes that it regulates, thus controlling their transcription. In this study, we provide evidence that *ideR* is an essential gene in *Mycobacterium tuberculosis*. *ideR* cannot normally be disrupted in this mycobacterium in the absence of a second functional copy of the gene. However, a rare *ideR* mutant was obtained in which the lethal effects of *ideR* inactivation were alleviated by a second-site suppressor mutation and which exhibited restricted iron assimilation capacity. Studies of this strain and a derivative in which IdeR expression was restored allowed us to identify phenotypic effects resulting from *ideR* inactivation. Using DNA microarrays, the iron-dependent transcriptional profiles of the wild-type, *ideR* mutant, and *ideR*-complemented mutant strains were analyzed, and the genes regulated by iron and IdeR were identified. These genes encode a variety of proteins, including putative transporters, proteins involved in siderophore synthesis and iron storage, members of the PE/PPE family, a membrane protein involved in virulence, transcriptional regulators, and enzymes involved in lipid metabolism.

Mycobacterium tuberculosis is an important human pathogen that causes serious infection in immunocompetent hosts but is particularly virulent in immunocompromised patients. Improved knowledge of mycobacterial pathogenesis is required to provide novel targets for intervention and to generate new vaccines.

One physiological condition known to be important in *M. tuberculosis* infection is the availability of iron. As is the case for most organisms, *M. tuberculosis* uses iron as a cofactor for enzymes that are involved in redox reactions and other essential functions, and it fails to grow in the absence of this metal (35). Free iron, however, is not readily available in the mammalian host, as it is mainly bound to high-affinity iron-binding proteins. Human serum is tuberculostatic, and this effect can be reversed by the addition of iron (23). On the other hand, abnormally high iron levels in *M. tuberculosis*-infected humans are associated with exacerbation of the disease (8, 14). In response to iron limitation, *M. tuberculosis*, like many other bacteria, produces high-affinity iron chelators, i.e., siderophores, which in mycobacteria are defined as mycobactins. One type of mycobactin remains cell associated, while the second (referred to as water-soluble mycobactin, carboxymycobactin, or exochelin [9, 15, 33]) has a shorter alkyl substitution, is more hydrophilic, and is released in the extracellular medium (15, 32). Supporting the concept that iron acquisition in the host is essential for virulence, failure to produce mycobactin results in defective bacillary multiplication in macrophages (9).

In addition to possessing the ability to acquire iron in the host, successful pathogens and essentially all aerobic organisms must carefully control the levels of intracellular iron. Failure to regulate this amount of iron in the cell could be lethal due to the ability of this metal to catalyze the production of toxic oxygen radicals in the presence of oxygen (21). Prokaryotes largely regulate intracellular iron levels by controlling its uptake (20). This is done by modifying the transcription of genes involved in iron acquisition, depending on the iron levels in the cell. Proteins that sense the levels of intracellular iron respond accordingly by modulating gene expression. *M. tuberculosis* contains four potential iron-dependent regulators belonging to two different families of metalloregulatory proteins. Two genes, *furA* and *furB*, encode proteins of the Fur (ferric uptake regulator) family, while IdeR and SirR are members of the DtxR (diphtheria toxin repressor) family (18, 26, 36). IdeR is the only one of these mycobacterial proteins that has been well characterized as to structure and function. It is a metal and DNA binding protein (11, 12, 30, 36). Like Fur and DtxR, IdeR binds iron and then interacts with a specific sequence in the operator regions of iron-regulated genes to control their transcription (10, 16, 34). The role of IdeR in iron regulation was first demonstrated in the nonvirulent mycobacterium *Mycobacterium smegmatis*, in which IdeR was found to be responsible for iron-dependent siderophore repression (11). The present study investigates the function of IdeR in *M. tuberculosis* and provides evidence that *ideR* is an essential gene in this mycobacterium. We analyzed the role of IdeR in iron-depen-

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dent gene expression and examined the requirement for IdeR in iron-dependent siderophore production and oxidative stress response.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* JM109 (43) was routinely used in DNA-cloning procedures. *M. tuberculosis* strain H37Rv (American Type Culture Collection) was maintained in Middlebrook 7H9 broth or on 7H10 agar (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% albumin-dextrose-NaCl complex (ADN) (22). When media with defined amounts of iron were needed, 7H9 and 7H10 were prepared omitting ferric ammonium citrate. We refer to these media as reconstituted 7H9 (r7H9) or r7H10. These media were subsequently supplemented with the desired amount of iron in the form of $FeCl_3$. Minimal medium (MM) was also used to grow cultures under defined iron conditions for the determination of mycobactin, growth curves (MM agar), and RNA extraction (MM broth). This medium contained 0.5% (wt/vol) asparagine, 0.5% (wt/vol) KH_2PO_4, 2% glycerol, 0.5 mg of $ZnCl_2$ liter^{-1}, 0.1 mg of $MnSO_4$ liter^{-1}, and 40 mg of $MgSO_4$ liter^{-1}. It was supplemented with the desired concentrations of $FeCl_3$, and in the case of the broth, 0.05% Tween 80 and 10% ADN were included. Where indicated, antibiotics were included at the following concentrations: kanamycin, 20 μg/ml; streptomycin, 20 μg/ml; and hygromycin, 150 μg/ml.

Construction of plasmids. The *M. tuberculosis* cosmid T144 was a kind gift of Stewart Cole of the Pasteur Institute (Paris, France). T144 was digested, and a 6.7-kb *Age*I fragment containing *ideR* was ligated to *Xba*I-digested pSM270, a suicide vector that carries *sacB* and a streptomycin resistance cassette in the plasmid backbone (24). A kanamycin resistance (Kan^r) cassette (*aph*) was then introduced at the unique *Apa*I site of *ideR* to generate pSM283. A 1.2-kb PCR fragment containing *ideR* and its promoter region was cloned into pMV306 to create pSM305. pMV306 carries a hygromycin resistance marker and the L5 integrase attachment site (*attP*) (38). DNA manipulations were performed by standard procedures.

Microarray analysis. DNA microarray analysis was used to measure the relative mRNA levels during growth of *M. tuberculosis* strains under low-iron and high-iron conditions. *M. tuberculosis* strains were grown in MM broth depleted of iron by treatment with Chelex 100 (Bio-Rad) or in the same medium supplemented with 50 μM $FeCl_3$. RNA extraction was performed as described previously (24).

Steps in *M. tuberculosis* DNA microarray gene expression analysis were performed as described by Schoolnik et al. (37). Briefly, each gene in *M. tuberculosis* H37Rv was amplified by PCR, and the DNA amplicons were printed onto poly-L-lysine-coated glass microscope slides to make the DNA microarray. cDNAs, made from two RNA samples labeled with either Cy3 or Cy5 (Amersham Pharmacia Biotech) fluorochrome, were hybridized to the microarray. The microarray was washed and then scanned using the GenePix 4000A (Axon Instruments). The intensities of the two dyes at each spot were quantified using SCANALYZE, written by M. Eisen at Stanford University and available at http://rana.stanford.edu/software. The overall reproducibility, both biological and technical, of the microarray experiments was evaluated using the Significance Analysis of Microarrays (SAM) program (37). Six DNA microarray experiments, which compared identical RNAs on the same array, were compared to six microarray experiments on three biological sample sets for each condition. The SAM algorithm was set for two-class unpaired analysis with 500 permutations and K-nearest imputer for missing data. Significantly regulated genes were selected by adjusting the delta value to give a false discovery rate below 1%. In each data set, all genes regulated 1.5-fold and greater were determined to be significant with a false discovery rate of less than 1%, indicating a high degree of reproducibility. Additional details of microarray methods are available as supplementary material at http://schoolniklab.stanford.edu/projects/tb.html.

Mycobactin determination. Mycobacterial strains were grown to mid-logarithmic phase in 7H9 medium, and 0.7 ml of culture was spread on MM agar containing the indicated concentrations of $FeCl_3$. After incubation at 37°C for 10 days, the bacteria were scraped from the plate. Subsequently, mycobactin was extracted in ethanol and chloroform and quantified as previously described (19).

Oxidative stress sensitivity assays. Growth inhibition by H_2O_2 and the superoxide generator plumbagin was tested in zone inhibition assays as previously described (24). Briefly, *M. tuberculosis* strains were grown to logarithmic phase (an optical density at 595 nm of 0.4) in 7H9 medium, and approximately 3×10^7 bacteria were plated on 7H10 medium and spread evenly. A 6.5-mm-diameter paper disk saturated with 10 μl of a solution of 600 mM hydrogen peroxide or 5 mM plumbagin was placed in the center of the plate. After incubation for 10 days, the bactericidal effect of each component was determined by measuring the diameter of the halo of growth inhibition. Triplicate platings were done in each experiment, and the experiment was repeated at least three times.

RESULTS

***ideR* is essential in *M. tuberculosis*.** To assess the role of IdeR in *M. tuberculosis*, we decided to inactivate *ideR* by allelic exchange. The system used for gene inactivation involves two steps of recombination and the use of the *Bacillus subtilis* levansucrase (*sacB*) as a counterselectable marker (29). In our modification of this system, a copy of the gene to be inactivated is disrupted by insertion of a kanamycin resistance cassette (*aph*) and cloned in pSM270, a suicide vector carrying *sacB* and a streptomycin resistance (Sm^r) marker (24) (Fig. 1A). A single-crossover event creates a Kan^r Sm^r intermediate that is sucrose sensitive (Suc^s) (Fig. 1B). Growth of the strain carrying the single crossover in nonselective medium allows a second recombination event between the wild-type gene in the chromosome and the mutated version on the integrated plasmid (Fig. 1B). This results in excision of intervening vector sequences including both *sacB* and the Sm^r marker. Therefore, the resulting colonies are Suc^r and Sm^s. Depending on which side of the kanamycin cassette the second crossover occurs on, the colonies obtained will have a wild-type or a mutated copy of the target gene and a Kan^s or Kan^r phenotype, respectively (Fig. 1C). If the sequences flanking the kanamycin cassette are of similar lengths, the second crossover should occur at similar frequencies on both sides.

The *ideR* gene disrupted by the *aph* cassette was cloned into the vector pSM270, generating pSM283, which was electroporated into *M. tuberculosis* H37Rv. Insertion of pSM283 by a single crossover at the *ideR* locus occurred in 100% (11 out of 11) of the Kan^r Sm^r colonies obtained, as determined by Southern blot analysis. However, after the second crossover, Suc^r Kan^r Sm^s mutant colonies were not obtained. This result suggested that inactivation of *ideR* could be lethal in *M. tuberculosis*. Since essential genes can be disrupted only in the presence of a second functional copy of the gene (17, 27, 28), we attempted to inactivate *ideR* by homologous recombination in a merodiploid as well as in a haploid strain. One strain (ST9) in which pSM283 had been inserted at the *ideR* locus by a single crossover (see above) was selected as the haploid strain. A merodiploid strain (ST17) was created by transformation of ST9 with an integrative plasmid (pSM305) carrying an intact *ideR* gene. Selection was made for hygromycin-resistant colonies, and insertion of pSM305 at the *attB* site of ST9 was confirmed by Southern blot analysis (data not shown). To select for double crossovers, ST9 and ST17 were grown to logarithmic phase in liquid medium and plated on sucrose-containing plates. No kanamycin was included so that both wild-type and *ideR* mutant colonies could be obtained. We reasoned that if IdeR had a role in regulating iron uptake in *M. tuberculosis*, as it does in *M. smegmatis* (11), an *ideR* mutant strain could be nonviable because it would face toxic iron overload. Therefore, reducing the amount of iron available in the medium might allow survival of an *ideR* mutant. For this reason, growth of the recombinant cultures and selection in sucrose were done under two conditions: in r7H9 medium with a high iron content (50 μM) or in the same medium with 10

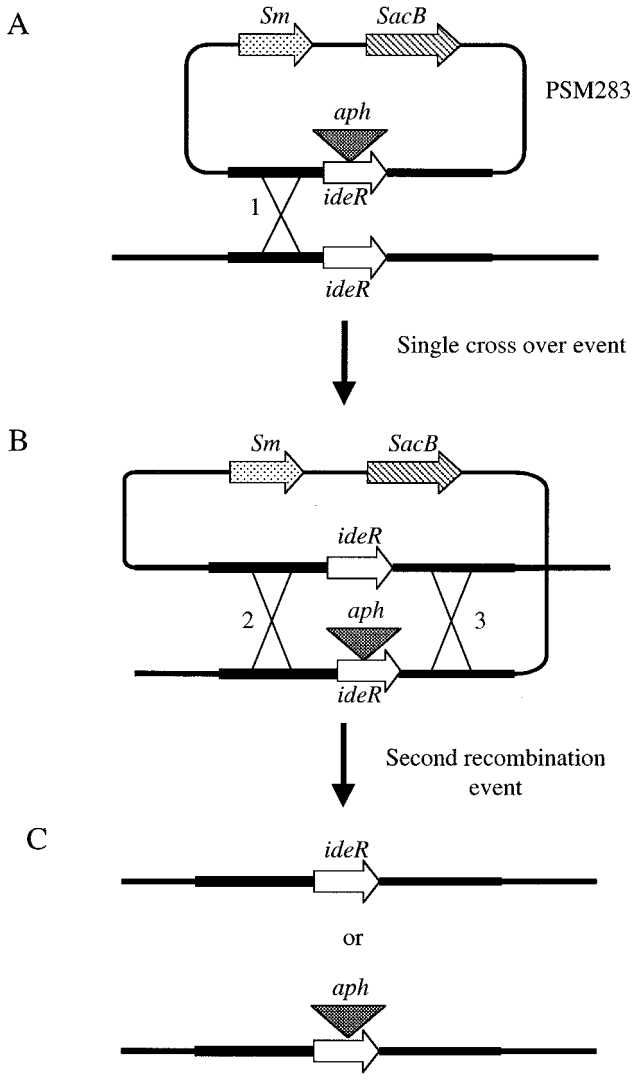

FIG. 1. Construction of single-crossover and double-crossover strains of *M. tuberculosis.* (A) The nonreplicative plasmid pSM283 was transformed into *M. tuberculosis*. Selection with kanamycin and streptomycin allows growth of strains in which pSM283 was inserted in the chromosome by a single crossover. The single crossover can occur at homologous regions on either side of the kanamycin cassette. Crossover at position 1 is illustrated. (B) A second recombination event between duplicated sequences leads to resolution of the duplication and loss of the plasmid sequences. (C) Depending on where the second recombination event occurs, i.e., interval 2 or interval 3, it can result in either restoration of the wild-type gene (interval 2) or a mutant strain (interval 3) in which allelic exchange has occurred.

μM $FeCl_3$ (previously shown to be the minimum concentration necessary to obtain normal-size colonies of *M. tuberculosis* on solid medium [data not shown]). In two independent experiments, 100 Suc^r colonies derived from the haploid or merodiploid strain were analyzed for kanamycin and streptomycin resistance. Recombinants without the disrupted *ideR* gene (Kan^s Sm^s) and *ideR* mutants (Kan^r Sm^s) resulting from double crossovers were obtained at similar frequencies in the Suc^r clones derived from the merodiploid strain (Table 1). However Suc^r Kan^r Sm^s clones were clearly selected against in the case of the haploid strain under both low- and high-iron conditions. Out of the total of 379 recombinants obtained in these experiments, only 3 had this phenotype (Table 1). The significant difference ($P < 0.0001$) observed between the frequencies of Kan^r Sm^s colonies isolated from strains that were haploid and merodiploid for *ideR* shows that the replacement of *ideR* with *ideR*::*aph* is essentially observed only when a second functional copy of the gene is present. These results strongly suggest that *ideR* is an essential gene in *M. tuberculosis.* The three Suc^r Kan^r Sm^s colonies derived from the haploid strain in these experiments (Table 1) were further analyzed. Replacement of the wild-type *ideR* by *ideR*::*aph* in these colonies was confirmed by Southern blot analysis (Fig. 2A). In agreement with this result, no IdeR protein was detected by Western blot analysis in protein extracts obtained from one of the recombinants, ST22 (Fig. 2B). We postulated that these rare mutants survived the lethal effects of *ideR* inactivation by acquisition of a suppressor mutation. In order to investigate the nature of this postulated mutation, ST22 was complemented with a single copy of *ideR* under the control of its own promoter, generating the strain ST52. Wild-type expression of the IdeR protein was restored in this strain (Fig. 2B). No significant difference was observed in the growth properties of H37Rv, ST22, and ST52 under standard culture conditions (Middlebrook 7H9 or 7H10 medium) (Fig. 3), except that cultures of ST22 showed a characteristic orange pigment (data not shown), the reasons for which will be discussed below. However, a significant difference was observed when the strains were tested for the ability to grow under low-iron conditions. The wild-type strain grew in low-iron medium at a growth rate that was comparable to that in iron-rich medium (Fig. 4A). In contrast, growth of the *ideR* mutant (Fig. 4B) and the complemented strain ST52 (Fig. 4C) was drastically affected by reducing the iron concentration in the medium. ST19, a Suc^r Kan^r Sm^s strain derived from the merodiploid strain in the previous experiment, was also tested. This strain has the original *ideR* inactivated by insertion of the *aph* cassette and a second copy of *ideR* at the *attB* site. ST19 is equivalent to ST52 but differs from it in that the recombination event resulting in inactivation of *ideR* was carried out in the presence of a second copy of *ideR*, thus avoiding selection for a possible suppressor mutation. As shown in Fig. 4D, ST19 was not deficient for growth under low-iron conditions and had the same capacity for iron assimilation under iron-limiting conditions as the wild-type strain. From these results, we conclude that strain ST22, in which the *ideR* mutation occurred in the absence of a second copy of *ideR*, was only able to survive due to a suppressor mutation associated with reduced availability of intracellular iron. This mutation was still present in the complemented strain, ST52, which shows the same phenotype of poor growth under low-iron conditions.

IdeR and regulation of iron-dependent gene expression. In previous studies, we have demonstrated that IdeR is a metal-dependent DNA binding protein that recognizes a specific promoter sequence, or "iron box" (10, 16, 34). We have also demonstrated deregulated expression of some *M. tuberculosis* promoters containing iron boxes in an *M. smegmatis ideR* mutant (34). A better knowledge of the regulatory function of IdeR would help us understand the essential role of this protein in *M. tuberculosis.* Thus, we reasoned that the *ideR* mutant strain ST22 could provide valuable information about IdeR-

TABLE 1. Frequency of *ideR* inactivation events in a merodiploid (ST17) versus a haploid (ST9) strain of *ideR*

Event	No. of transformants[a]							
	Merodiploid (ST17)				Haploid (ST9)			
	High iron		Low iron		High iron		Low iron	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Wild-type double crossover[b]	48	45	40	45	94	97	94	94
Mutant double crossover[c]	52	55	56	55	0	0	0	3

[a] Strains were plated in 8% sucrose in the absence of antibiotics. One hundred sucrose-resistant colonies from two independent experiments were tested for kanamycin and streptomycin resistance. The second crossover and selection in sucrose were allowed to occur in high-iron (50 μM) or low-iron (10 μM) concentrations. In these experiments, kanamycin-resistant, streptomycin-resistant colonies (spontaneous sucrose-resistant colonies presumably due to *sacB* mutations) occurred at a frequency of 6% or less. For the differences in the frequencies of mutant double crossover and wild-type double crossover between the merodiploid and the haploid strains, P was <0.0001; χ^2 or Fisher's exact test (two tailed) was used.
[b] Kanamycin-sensitive, streptomycin-sensitive colonies.
[c] Kanamycin-resistant, streptomycin-sensitive colonies.

regulated genes even though it contained a suppressor mutation. IdeR-dependent genes could be identified as those whose regulation in response to iron was altered in the *ideR* mutant in respect to the wild type and whose normal regulation was restored by the presence of IdeR in the complemented strain. For this purpose, we did global expression profiling with DNA microarrays comparing wild-type H37Rv with ST22 and ST52 strains. Genes whose expression was modulated by iron were identified by comparing the transcriptional profile of the wild-type strain grown under low- versus high-iron conditions. Genes that required iron and IdeR for regulation were identified by comparing the wild-type strain's high-iron expression profile with the expression profiles of mutant and complemented strains. Only high-iron RNA samples from the mutant and complemented strains were analyzed, since under high-iron conditions, the three strains have comparable growth rates and changes in gene expression would not be affected by growth rate differences. A total of 153 genes were found to be regulated by iron, and almost a third of them (51) were dependent on IdeR for regulation. For reasons of space, we tabulated genes that were affected (2.0-fold or more in Tables 2, 3, and 4 and 1.6 or more in Table 5). A list of all genes regulated at least 1.5-fold under the conditions assayed can be obtained at http://schoolniklab.stanford.edu/projects/tb.html, and complete data sets are available upon request. Table 2 shows genes that required IdeR for iron-dependent repression, as they were no longer repressed in the IdeR mutant under high-iron conditions. IdeR was also necessary for high levels of expression of some genes under high-iron conditions (Table 3). There were genes whose regulation was altered in both the

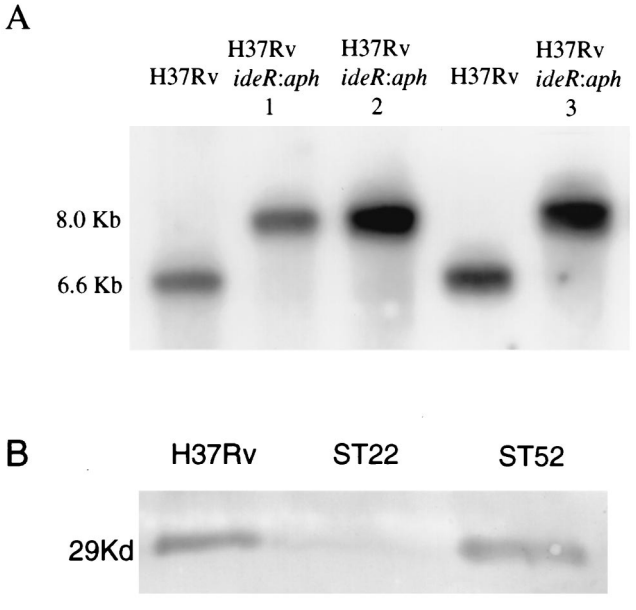

FIG. 2. Analysis of *ideR* mutant strains. (A) Chromosomal DNAs from the wild-type and the three *ideR* mutant colonies (1, 2, and 3) obtained were extracted, digested with *Age*I, and analyzed by Southern blotting with a ^{32}P-labeled probe corresponding to the 0.7-kb *ideR*. A 6.7-kb *Age*I fragment was detected with the *ideR* probe in the wild-type strain. The replacement of *ideR* by *ideR*::*aph* increases the size of the *Age*I fragment to 8.0 kb. (B). Whole cell protein extracts were prepared from wild-type H37Rv, the *ideR* mutant ST22, and the complemented strain ST52; 50 μg of protein extracts was loaded in each lane. IdeR was detected by immunoblot analysis using an anti-IdeR polyclonal antiserum as previously described (11). Purified IdeR migrates at a corresponding molecular mass of 29 kDa (data not shown).

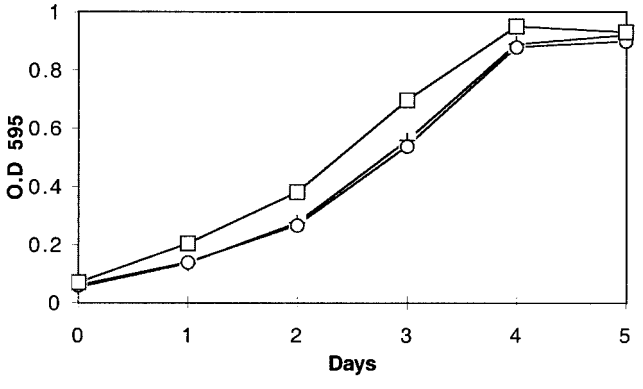

FIG. 3. Growth of *M. tuberculosis* strains. *M. tuberculosis* strains were grown in Middlebrook 7H9 medium, and at the indicated times, bacterial growth was monitored by measuring the change in optical density at 595 nm (O.D. 595). The experiment was performed at least three times, and one representative experiment is shown. +, H37Rv; ○, ST22; □, ST52.

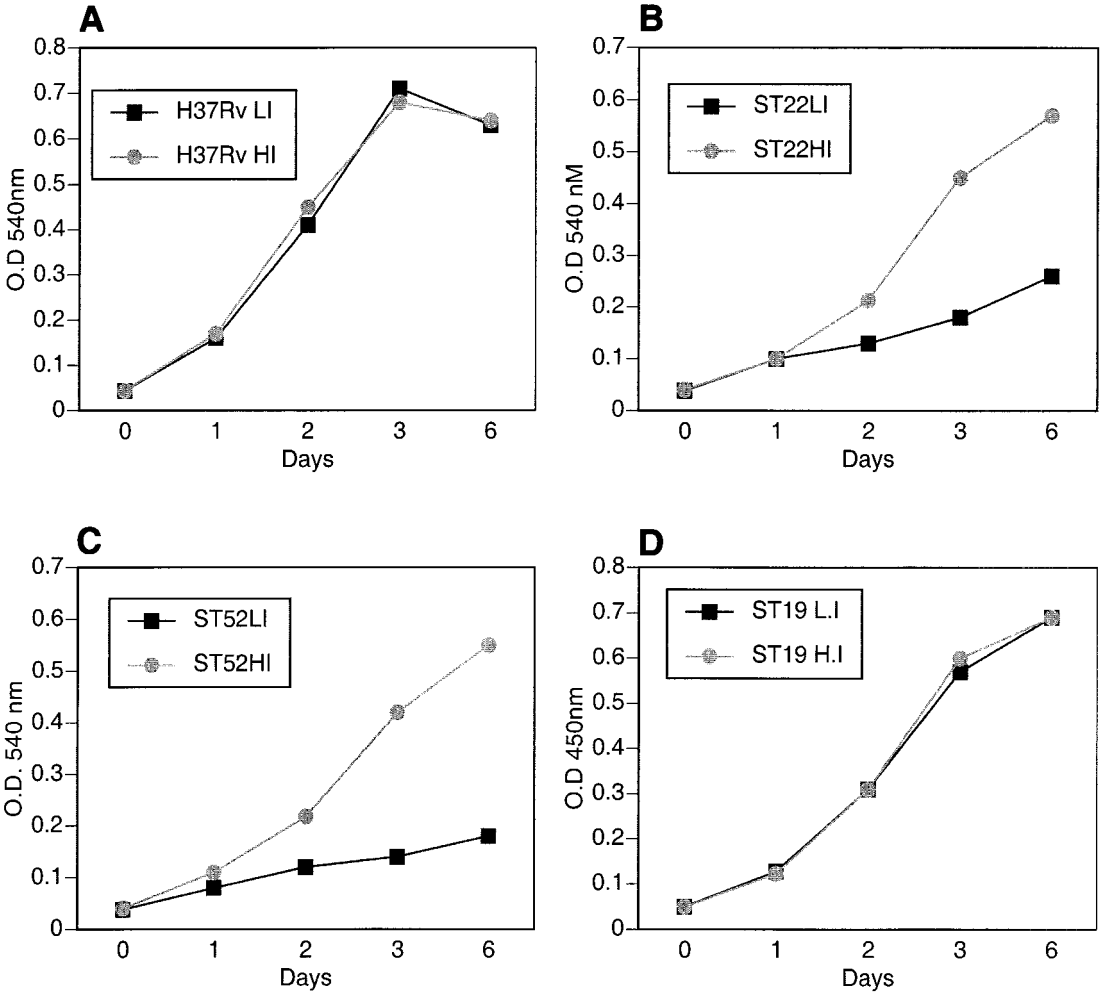

FIG. 4. Iron requirements for growth of *M. tuberculosis* strains. Wild-type H37Rv (A), ST22 (*ideR*::*aph*) (B), ST52 (*ideR*-complemented mutant) (C), and ST19 (D) were grown in iron-deficient liquid medium to logarithmic phase and then diluted in MM plus 10% ADN containing 2 (■) or 50 μM (●) $FeCl_3$. Growth was monitored by changes in optical density. The experiment was performed at least three times, and the results shown are from one representative experiment.

ideR mutant and the complemented strain. These included Rv0116c and Rv0587, whose normally repressed levels under high-iron conditions were high in ST22 and ST52, as well as *bfrB*, which was not expressed in ST22 or ST52 under high-iron conditions at the same level as in the wild type. Regulation of these genes was probably affected by the suppressor mutation present in both of the strains.

Iron-dependent but IdeR-independent genes are listed in Tables 4 and 5. This group of genes was negatively (Table 4) or positively (Table 5) regulated by iron in H37Rv, but its regulation was not affected by the *ideR* mutation, as none of the genes were found to be deregulated in the *ideR* mutant strain (data not shown).

All the IdeR-regulated genes identified in the microarray analysis listed in Table 2 have sequences resembling DtxR/IdeR binding sites (iron boxes) in their upstream regions. In several cases, one or two iron boxes are found in the intergenic region between two divergently transcribed genes or preceding two or more open reading frames (ORFs) which appear to be organized as an operon. These sequences were identified previously in computer analyses. IdeR was shown to bind to nine of the iron boxes, which are operator sites for the following genes: Rv2122c (*hisE*/*irg-1*), Rv2123 (*irg-2*), Rv3402, Rv1876 (*bfrA*), *bfd*, Rv 2386c (*mbtI*), Rv2384 (*mbtA*), Rv2383c (*mbtB*), Rv2382c (*mbtC*), Rv 3281c (*mbtD*), Rv2380c (*mbtE*), Rv2379c (*mbtF*), Rv2378c (*mbtG*), Rv 2377c (*mbtH*), and Rv2385 (*lipK*) (16, 34). Therefore, the results obtained in the microarray assays presented here and our previous biochemical studies of the interaction of IdeR with these iron boxes support each other and allow us now to better define the consensus binding site for IdeR. Figure 5 shows a comparison of all the iron boxes found in the regulatory regions of the IdeR-controlled genes that we have identified and the consensus sequence deduced for the 19-bp core region of the IdeR binding site.

Iron-dependent siderophore production is dependent on IdeR. Our laboratory has previously demonstrated that *mbtB* and *mbtI*, two of the 10 genes in the mycobactin synthesis gene cluster, are induced in *M. tuberculosis* under iron-restrictive conditions and during infection of macrophages (16). In addition, we have shown the presence of two IdeR operators in this

TABLE 2. Iron- and IdeR-repressed genes[a]

Rv no.	Gene	mRNA ratio			Gene product[b]
		WT LI/HI	ST22 HI/WT HI	ST52 HI/WT HI	
0116c		2.3 ± 0.3	2.1 ± 0.3	2.7 ± 0.6	CHMP
0282		3.9 ± 0.5	3.2 ± 0.3	1.0	CHP
0283		2.9 ± 0.6	2.9 ± 0.5	1.0	CHMP
0284		4.1 ± 0.6	3.7 ± 0.5	1.0	CHMP
0285		3.0 ± 0.5	2.7 ± 0.1	1.0	PE subfamily
0286		2.6 ± 0.3	2.3 ± 0.2	1.0	PPE subfamily
0287		4.0 ± 0.6	4.9 ± 0.9	1.0	Probable transcriptional regulator; PE subfamily
0288		3.5 ± 0.4	4.2 ± 0.1	1.0	CHP; ESAT-6 family
0289		3.2 ± 0.2	2.7 ± 0.3	1.0	CHP
0290		2.0 ± 0.2	1.9 ± 0.2	1.0	Unknown
0291		3.0 ± 0.3	2.3 ± 0.4	1.0	Probable secreted protease
0292		2.7 ± 0.3	1.8 ± 0.2	1.0	Unknown
0766c		3.3 ± 0.4	2.9 ± 0.3	1.0	Cytochrome P-450
0450c	*mmpL4*	2.1 ± 0.3	2.5 ± 0.1	1.0	Conserved large membrane protein
0451c	*mmpS4*	3.1 ± 0.4	3.6 ± 0.6	1.0	Conserved small membrane protein
0587	*yrbE2A*	2.2 ± 0.2	2.1 ± 0.2	2.5 ± 0.3	Part of the *mce2* operon
1343c		2.5 ± 0.5	2.1 ± 0.2	1.0	CHP
1344		3.9 ± 0.6	3.2 ± 0.4	1.0	Probable acyl carrier protein
1345	*fadD33*	3.4 ± 0.4	2.7 ± 0.5	1.0	Acyl-CoA synthase
1346	*fadE14*	4.9 ± 0.9	3.3 ± 0.5	1.0	Acyl-CoA dehydrogenase
1347c*		7.9 ± 1.7	6.4 ± 0.5	1.0	IucB-like protein
1348*		5.1 ± 0.7	5.3 ± 1.0	1.0	YbtP-like protein
1349*		4.3 ± 1.2	4.6 ± 0.7	1.0	YbtQ-like protein
1519		3.7 ± 0.4	2.9 ± .03	1.0	CHP
2122c*	*hisE*	3.1 ± 0.3	2.5 ± 0.4	1.0	Phosphoribosyl-AMP cyclohydrolase
2123		10.1 ± 2.1	10.1 ± 3.9	1.0	PPE subfamily
2377c	*mbtH*	5.4 ± 1.9	5.6 ± 1.5	1.0	Mycobactin synthesis
2378c	*mbtG*	5.5 ± 1.5	4.7 ± 0.8	1.0	Lysine-*N*-oxygenase mycobactin synthesis
2379c	*mbtF*	3.6 ± 0.7	2.4 ± 0.4	1.0	Peptide synthase; mycobactin synthesis
2380c	*mbtE*	7.1 ± 0.9	5.4 ± 1.1	1.0	Peptide synthase; mycobactin synthesis
2381c	*mbtD*	9.9 ± 1.8	7.5 ± 0.9	1.8 ± 0.1	Polyketide synthase; mycobactin synthesis
2382c	*mbtC*	5.3 ± 0.4	3.6 ± 0.8	1.0	Polyketide synthase; mycobactin synthesis
2383c	*mbtB*	5.4 ± 0.8	3.9 ± 0.8	1.0	Peptide synthase; mycobactin synthesis
2384	*mbtA*	1.9 ± 0.3	1.8 ± 0.2	1.0	Salicylate-AMP ligase; mycobactin synthesis
2385	*lipK*	3.6 ± 0.6	3.2 ± 0.6	1.0	Probable acetyl-hydrolase
2386c	*mbtI (trpE2)*	8.6 ± 1.8	9.3 ± 1.8	1.9 ± 0.2	Isochorismate synthase Mycobactin synthesis
3402c*		9.7 ± 2.5	5.1 ± 1.0	1.0	EryCIV-like protein
3403c		5.6 ± 1.0	3.0 ± 0.3	1.0	Unknown
3839		19.3 ± 7.0	14.1 ± 3.7	1.0	Unknown
3840		2.6 ± 0.5	6.7 ± 0.5	1.0	Probable transcriptional regulator

[a] A DNA microarray was used to measure mRNA levels in cultures of *M. tuberculosis* H37Rv (WT) during exponential growth under iron-deficient (2 μM $FeCl_3$) (LI) or -sufficient (50 μM $FeCl_3$) (HI) conditions and in exponential cultures of strains ST22 and ST52 grown in iron-sufficient medium. Differences in gene-specific RNA levels of twofold or more are listed. Genes contiguous to included genes and with a ratio of >1.6 after the standard deviation was subtracted are also listed. Genes that required IdeR for repression were repressed under high-iron conditions in the wild-type strain (WT LI/HI > 2.0), derepressed in the *ideR* mutant ST22 under high-iron conditions (ST22 HI/WT HI > 2.0), and repressed in the mutant complemented strain ST52 under high-iron conditions to the same levels observed in the wild type under high iron conditions (ST52 HI/WT HI = 1.0). The genes are annotated as described by the Pasteur Institute on TUBERCULIST (http://genolist.pasteur.fr/Tuberculist), except for those indicated by an asterisk. Those genes are annotated based on individual BLAST searches. Genes that are separated by <50 bp and probably coregulated are grouped.

[b] CHP, conserved hypothetical protein. CHMP, conserved hypothetical membrane protein; CoA, coenzyme A.

TABLE 3. Genes induced by iron and IdeR[a]

Rv no.	Gene	mRNA ratio			Gene product
		WT HI/LI induced	WT HI/ST22 HI	WT HI/ST52 HI	
0009	*ppiA*	2.7 ± 0.1	3.0 ± 0.3	1.0	Peptidyl-prolyn-*cis-trans* isomerase
0338c		2.8 ± 0.2	2.4 ± 0.4	1.0	Iron-sulfur protein
1552	*bfrA*	2.1 ± 0.1	2.6 ± 0.2	1.0	Bacterioferritin
3841	*bfrB*	11.5 ± 1.8	4.3 ± 0.7	3.8 ± 0.6	Ferritin-like protein

[a] A DNA microarray was used to measure mRNA levels in cultures of *M. tuberculosis* H37Rv during exponential growth under iron-deficient (2 μM $FeCl_3$) (LI) or -sufficient (50 μM $FeCl_3$) (HI) conditions and in exponential cultures of strains ST22 and ST52 grown in iron-sufficient medium. Differences in gene-specific mRNA levels of twofold and higher are listed. Iron- and IdeR-induced genes were identified as genes induced under high-iron conditions in the wild-type strain (WT HI/LI > 2), not induced under high-iron conditions in the *ideR* mutant ST22 (WT HI/ST22 HI > 2), and induced under high-iron conditions in the complemented strain ST52 to levels comparable to those in the wild type under high-iron conditions (WT HI/ST52 HI = 1.0). The Genes are annotated as described by the Pasteur Institute on TUBERCULIST (http://genolist.pasteur.fr/Tuberculist).

gene cluster: one upstream of *mbtI* and one in the promoter region of the divergently transcribed *mbtA* and *mbtB* to *-H* (16). Expression of each of the 10 genes present in the mycobactin synthesis gene cluster is deregulated in the absence of IdeR (Table 2). The induction of *mbtB* and *mbtI* observed here was less than that observed by Gold et al. This could be due to the fact that cultures were starved for iron for a longer time in the latter work. To validate the observations from the DNA microarray analysis, iron-dependent regulation of mycobactin production was tested by determining the amounts of cell-

TABLE 4. IdeR-independent, iron-repressed genes[a]

Rv no.	Gene	mRNA ratio (WT LI/WT HI)	Gene product[b]
0279c	PE-PGRS	2.2 ± 0.2	PE-PGRS protein
0464c		2.2 ± 0.2	CHP
0465c		4.3 ± 0.7	Probable transcriptional regulator (PbsX/Xre family)
0467	*icl*	3.5 ± 0.4	Isocitrate lyase
0676c	*mmpL5*	2.8 ± 0.2	Conserved large membrane protein
0677c	*mmpS5*	3.2 ± 0.3	Conserved small membrane protein
0692		2.7 ± 0.2	CHP
0693	*pqqE*	2.5 ± 0.3	Coenzyme PQQ synthesis protein E
0694	*lldD1*	2.3 ± 0.2	L-Lactate dehydrogenase (cytochrome)
1169c		2.2 ± 0.2	PE protein
1184c		2.2 ± 0.1	CHP
1195		2.2 ± 0.2	PE protein
1393c		2.4 ± 0.3	FAD-containing monooxygenase
1461		4.7 ± 0.4	CHP
1462		3.4 ± 0.6	CHP
1463		3.3 ± 0.5	Probable ABC transporter
1464		3.0 ± 0.3	NifS-like protein
1465*		2.6 ± 0.5	NifU-like protein
1466		3.2 ± 0.6	CHP
1520		2.1 ± 0.2	Probable glycosyltransferase
2621c		2.4 ± 0.3	Probable transcriptional regulator
2794c		2.7 ± 0.2	CH; similar to proteins involved in vibriobactin and enterobactin synthesis
3229c	*desA3*	5.0 ± 2.7	Acyl[ACP] desaturase
3230c		2.5 ± 0.5	Probable oxygenase
3614c		2.1 ± 0.1	CHP
3854c	*ethA*	4.4 ± 0.4	Probable monooxygenase

[a] A DNA microarray was used to measure mRNA levels in cultures of *M. tuberculosis* H37Rv during exponential growth under iron-deficient (2 μM $FeCl_3$) (LI) or -sufficient (50 μM $FeCl_3$) (HI) conditions and in exponential cultures of strains ST22 and ST52 in iron-sufficient medium. Differences in gene-specific RNA levels of twofold or more are listed. Genes contiguous to included genes and with a ratio of > 1.6 after the standard deviation was subtracted are also listed. Iron-dependent, IdeR-independent genes were derepressed under low-iron conditions in the wild-type (WT LI/HI > 2) and were not affected in their repression under high-iron conditions in the *ideR* mutant. All these genes showed a WT HI/ST22 HI ratio of 1.0 (not shown). The genes are annotated as described by the Pasteur Institute on TUBERCULIST (http://genolist.pasteur.fr/Tuberculist), except for that indicated by an asterisk. That gene is annotated based on an individual BLAST search. Genes that are separated by <50 bp and are probably coregulated are grouped.

[b] CHP, conserved hypothetical protein; CHMP, conserved hypothetical membrane protein.

TABLE 5. IdeR-independent iron-induced genes[a]

Rv no.	Gene	mRNA ratio (WT HI/LI)	Gene product[b]
0706	*rplV*	1.6 ± 0.1	50S ribosomal protein L22
1252c	*IprE*	1.6 ± 0.1	Lipoprotein
1305	*atpE*	1.6 ± 0.1	ATP synthase c chain
1908c	*KatG*	1.7 ± 0.2	Catalase-peroxidase
1943c		1.7 ± 0.1	CHP
2526		1.6 ± 0.1	CHP
2549c		1.6 ± 0.1	Unknown
2550c		2.0 ± 0.2	Unknown
2741	*PE-PGRS*	2.0 ± 0.1	PE-PGRS subfamily
2927c		1.6 ± 0.1	CHP
3075c		1.7 ± 0.2	CHP
3145	*nuoA*	1.7 ± 0.3	NADH dehydrogenase chain A
3146	*nuoB*	1.8 ± 0.1	NADH dehydrogenase chain B
3147	*nuoC*	1.6 ± 0.2	NADH dehydrogenase chain C
3148	*nuoD*	1.7 ± 0.2	NADH dehydrogenase chain D
3152	*nuoH*	1.8 ± 0.1	NADH dehydrogenase chain H
3153	*nuoI*	1.6 ± 0.1	NADH dehydrogenase chain I
3155	*nuoK*	1.6 ± 0.2	NADH dehydrogenase chain K
3156	*nuoL*	1.7 ± 0.2	NADH dehydrogenase chain L
3157	*nuoM*	1.6 ± 0.1	NADH dehydrogenase chain M
3158	*nuoN*	1.6 ± 0.1	NADH dehydrogenase chain N
3246	*mtrA*	2.0 ± 0.3	Two-component response regulator
3394		1.7 ± 0.2	Unknown

[a] A DNA microarray was used to measure mRNA levels in cultures of *M. tuberculosis* H37Rv during exponential growth under iron-deficient (2 μM $FeCl_3$) (LI) or -sufficient (50 μM $FeCl_3$) (HI) conditions and in exponential cultures of strains ST22 and ST52 in iron-sufficient medium. Genes induced by 1.6-fold or more are listed. Iron-dependent, IdeR-independent genes were induced under high-iron conditions in the wild type (WT HI/LI > 1.6), and their expression under high-iron conditions was not affected in the *ideR* mutant. All these genes showed a WT HI/ST22 HI ratio of 1.0 (not shown). The genes are annotated as described by the Pasteur Institute on TUBERCULIST (*http://genolist.pasteur.fr/Tuberculist*). Genes that are separated by <50 bp and are probably coregulated are grouped.

[b] CHP, conserved hypothetical protein; CHMP, conserved hypothetical membrane protein.

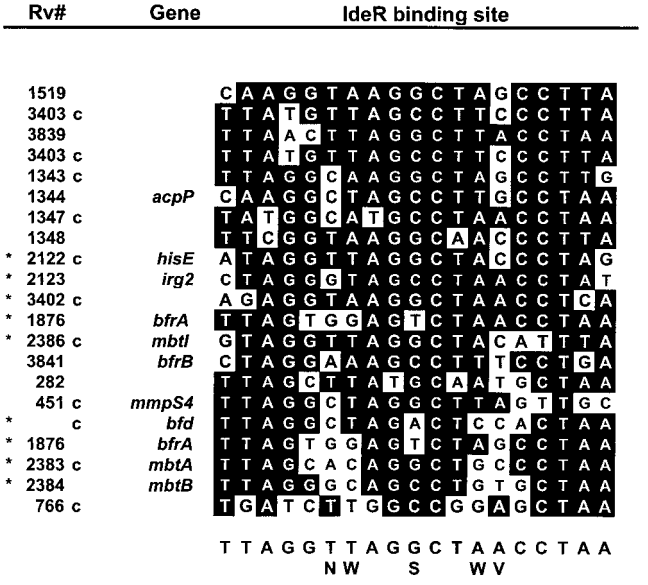

FIG. 5. Comparison of the known IdeR operator sequences from *M. tuberculosis*. The 19-bp consensus sequence was derived by comparing the sequences of the promoter-operator sequences in all of the IdeR-regulated genes identified from Tables 2 and 3 and is shown below the sequences. In cases where one IdeR binding site is found upstream of the first gene in a group of contiguous genes, only the first ORF (Rv) is cited. N = A, C, G, or T; W = A or T; S = C or G; V = A, C, or G. Solid boxes (black) indicate a predominance of one nucleotide in the IdeR binding site sequences. Actual binding of the IdeR protein to the sequences marked with asterisks has been demonstrated by gel shift and DNase protection assays in previous studies (16, 34).

associated mycobactin produced by the wild-type, ST22 (*ideR*), and ST52 (*ideR*-complemented) strains when grown under low- and high-iron conditions. The wild-type and complemented strains repressed mycobactin production under high-iron conditions, while the *ideR* mutant strain failed to repress mycobactin synthesis and accumulated this siderophore in an iron-independent manner (Fig. 6). Accumulated ferric mycobactin is likely to account for the orange pigment showed by this strain when grown in iron-rich medium. From these results, we conclude that IdeR is the main regulator of mycobactin production in *M. tuberculosis.*

The repression of mycobactin genes and mycobactin production by high levels of iron in the complemented strain is contradictory to the postulated action of the suppressor mutation in iron assimilation. If this mutation affects iron assimilation, the available iron in the complemented strain should be reduced and expression of iron-regulated genes should be affected. It is possible that 50 μM $FeCl_3$, used in these experiments as high-iron conditions, was sufficient to allow efficient iron uptake despite the suppressor mutation. As shown in Fig. 4, this concentration was sufficient to allow normal growth of the IdeR mutant and complemented strains. However, an effect of iron deficiency in repression of mycobactin biosynthetic genes, as well as other iron-dependent genes, could be manifested under lower iron concentrations. For this reason, we compared repression of mycobactin production in the wild-type and the complemented strain over a range of iron concentrations (Fig. 7). Even though the concentration of iron required to achieve total repression of mycobactin varied from experiment to experiment, probably due to variations in the

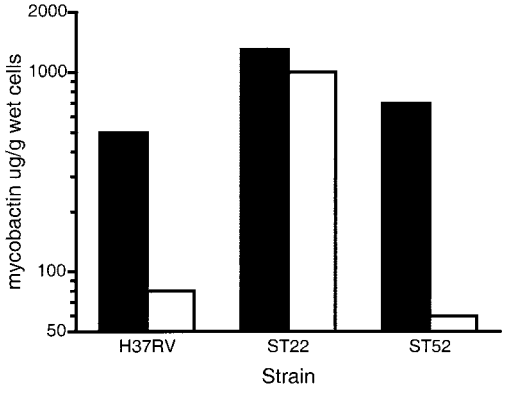

FIG. 6. Regulation of mycobactin production. Levels of cell-associated mycobactin extracted from wild-type H37Rv, ST22 (*ideR*::*aph*), and ST52 (*ideR*-complemented) strains cultured under low-iron (10 μM; solid bars) or high-iron (50 μM; open bars) conditions. The experiment was repeated at least three times, and the values shown illustrate one representative experiment.

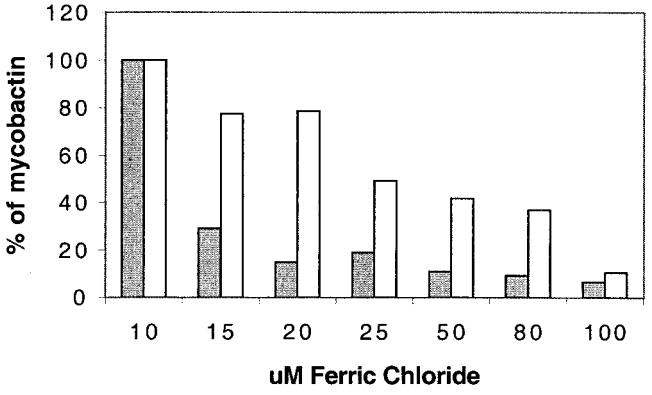

FIG. 7. Iron and mycobactin repression. H37Rv and ST52 were cultivated in MM agar with increasing concentrations of $FeCl_3$. Mycobactin was extracted from each culture and quantified. The data are expressed as percentages of mycobactin produced in each concentration of iron, 100% being the amount of mycobactin produced under the lowest iron concentration, which was 10 μM $FeCl_3$. Shaded bars, H37Rv; open bars, ST52. The experiment was repeated five times, and the values shown illustrate one representative experiment.

intracellular iron contents of plated cells and the agar medium, we consistently observed that more $FeCl_3$ was required to repress mycobactin production in the complemented strain than in the wild-type strain. Maximum repression of mycobactin in the wild-type strain required 20 to 25 μM $FeCl_3$, whereas the same effect in the complemented strain required 50 to 100 μM $FeCl_3$. A representative experiment is presented in Fig. 7. This result agrees with the iron deficiency phenotype of strains ST22 and ST52 and supports the interpretation that a suppressor mutation associated with lower iron assimilation alleviated the effect of the IdeR mutation.

IdeR is necessary for an efficient response to oxidative stress. Inactivation of IdeR in *M. smegmatis* results in increased sensitivity to oxidative stress, apparently resulting from reduced levels of catalase-peroxidase and superoxide dismutase in the mutant strain (11). Therefore, we compared the sensitivities of the wild type, the *M. tuberculosis ideR* mutant, and the complemented mutant strain to H_2O_2 and the superoxide generator plumbagin. The *ideR* mutant strain was found to be significantly more sensitive to both H_2O_2 and plumbagin than the wild-type strain (Fig. 8). This phenotype was due to inactivation of *ideR*, since resistance to oxidative stress was restored to wild-type levels in the complemented strain. However, in contrast to previous findings with *M. smegmatis*, neither expression of *katG* and *sodA* nor the activity of the enzymes they encode is affected by IdeR inactivation (data not shown).

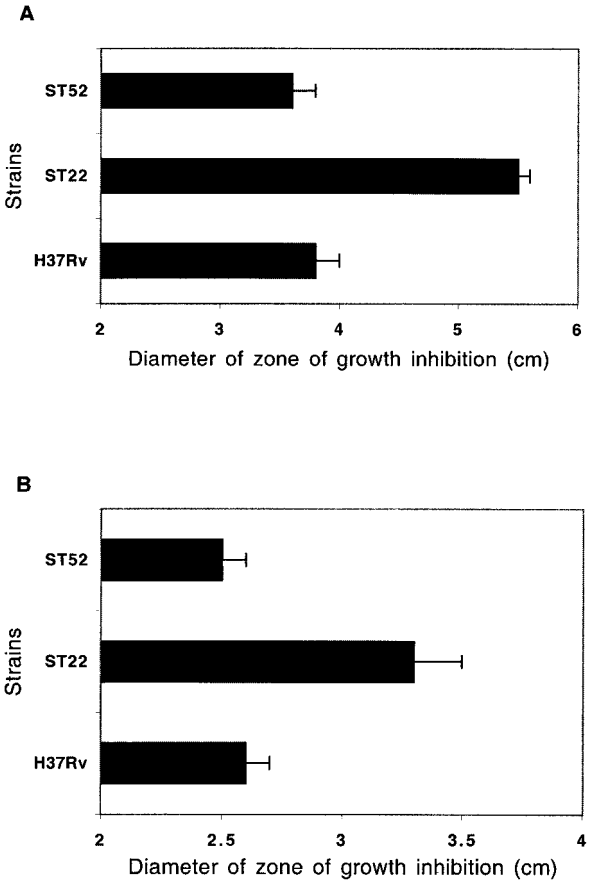

FIG. 8. Sensitivity to oxidative stress. Shown are the diameters of the zones of growth inhibition produced in the presence of 600 mM hydrogen peroxide (A) and 5 mM plumbagin (B). Wild-type H37Rv, ST22 (*ideR*::*aph*), and ST52 (*ideR* complemented) were grown in 7H9 medium and plated in 7H10 medium. The values represent means plus standard deviations (the experiments were performed in triplicate).

DISCUSSION

In this study, we have investigated the role of IdeR, a regulator of genes responding to iron, in *M. tuberculosis*. Initial unsuccessful attempts to create an *ideR* mutant by allelic exchange using a two-step homologous-recombination strategy suggested that this gene was essential. However, construction of a merodiploid strain containing an integrated copy of *ideR* allowed inactivation of the gene, providing formal proof for its essentiality. The requirement for a major iron regulator like *ideR* is not unique to *M. tuberculosis*, since null mutations in *fur*, a functional homologue of *ideR*, are known to be lethal in several species of *Pseudomonas* (40), *Vibrio* (39), and *Neisseria* (2). *ideR* is not essential in all mycobacteria, as we previously inactivated this gene in the saprophyte *M. smegmatis* (11). It will be interesting to determine whether the requirement for IdeR is a feature shared by other pathogenic mycobacteria. The functions of IdeR that make it an essential protein in *M. tuberculosis* are unknown. In this study, we isolated a rare recombinant (ST22) rescued from the lethal effects of *ideR* inactivation by a suppressor mutation. This strain exhibits restricted intracellular iron availability (Fig. 4B and 7). This suggests that the essential role of IdeR is related to regulation of intracellular levels of iron. If the *ideR* mutation results in unregulated iron uptake leading to iron toxicity, a suppressor mutation which lowers iron availability could prevent cell death.

Restoring IdeR expression in ST22 by complementation with an integrative copy of *ideR* allowed us to identify phenotypes resulting directly from *ideR* inactivation. We identified iron-modulated genes that were deregulated in the mutant

strain but not in the complemented strain by comparing the levels of mRNA of all *M. tuberculosis* ORFs in the wild-type, mutant, and complemented mutant strains using DNA microarray technology. This is the first study that addresses the global genetic response of *M. tuberculosis* to different iron levels. In previous studies, several *M. tuberculosis* iron-regulated proteins were identified by one- (4) or two-dimensional (42) gel electrophoresis combined with N-terminal sequencing or mass spectrometry, respectively. Our results confirmed iron-induced transcription of *ppiA*, encoding a peptidyl-prolyl transisomerase previously found to be reduced under low-iron conditions (42). Genes encoding the other proteins found in those studies to be modulated by iron did not come up in our array assay. It is possible that iron-dependent expression of those proteins is controlled posttranscriptionally.

The IdeR-controlled genes identified in this study encode proteins with diverse putative functions, including transporters (Rv0282, Rv0283, and Rv0284), enzymes involved in lipid metabolism (Rv1344, Rv1345, and Rv1347), members of the glycine-rich PE/PPE protein family (Rv0285, Rv0286, and Rv2123), and MmpL4 and MmpS4, which belong to a group of conserved membrane proteins in *M. tuberculosis* sharing sequence and structural similarities (6). MmpL4 was identified by signature-tagged transposon mutagenesis as a potential virulence factor of *M. tuberculosis* (5). However, as expected, the largest group of genes regulated by IdeR encode proteins that have or could have a function in iron metabolism. Included in this group are the 10 *mbt* genes encoding the enzymes for mycobactin synthesis (31); *bfrA*, encoding a putative bacterioferritin; Rv1348 and Rv1349, encoding homologs of YbtP and YbtQ, which are ABC transporters required for iron uptake in *Yersinia pestis* (13); Rv1347, encoding a protein similar (29% identity in a 161-amino-acid overlap) to the aerobactin synthesis protein IucB of *Shigella boydii;* and several membrane proteins which might have roles in iron transport. As a validation of the results of the DNA microarray assays, we measured mycobactin production and showed that the repression of mycobactin production under high-iron conditions is indeed dependent on IdeR (Fig. 6). These results are consistent with our previous observations demonstrating functional IdeR binding sites in the promoter regions of *mbt* genes and induction of these genes under low-iron conditions (16). We also demonstrated binding of IdeR to the regulatory region of *bfrA* and showed that under high-iron conditions *bfrA* is transcribed from a promoter that is activated in vitro by iron and binding of IdeR (16). The induction of *bfrA* by high-iron conditions in the wild-type and complemented strains but not in the *ideR* mutant confirms the role of IdeR as a positive regulator of *bfrA* expression.

Genes regulated by iron but independent of IdeR were also identified (Tables 4 and 5). Genes encoding proteins involved in intermediate metabolism, aerobic growth, transcriptional regulation, iron utilization, and transporters are part of this group. Notably, 10 out of the 14 genes encoding the polypeptide components of the NADH dehydrogenase were modestly induced by high-iron conditions. Since [Fe-S] clusters are evolutionarily conserved prosthetic groups in NADH dehydrogenases (41), iron available for incorporation into these groups might serve as a signal for increased synthesis of NADH dehydrogenase polypeptides. Interestingly, *ethA*, encoding a monooxygenase that has been postulated to activate the antituberculosis agent ethionamide (1, 7), was found to be induced under low-iron conditions. In view of this result, it will be important to examine the relative sensitivities of *M. tuberculosis* to ethionamide under different iron conditions. *mtrA*, encoding the response regulator of the two-component system MtrA-MtrB, which has been shown to be essential in *M. tuberculosis* (44), was induced twofold under high-iron conditions. Also noteworthy was the induction of *icl* under iron deficiency. *icl*, also known as *aceA*, encoding isocitrate lyase, is important for the survival of *M. tuberculosis* during the persistence phase of infection in mice (25). Since *icl* is also necessary for survival in activated macrophages, this gene has been postulated to be required once a cell-mediated immune response is induced (25). One of the responses of mononuclear phagocytes to activation by gamma interferon is downregulation of the transferrin receptor, the major source of iron for the cell, a response that could cause iron deficiency for the infecting bacteria (3). Furthermore, we find that genes that are upregulated in vitro under iron deficiency are also induced during macrophage infection, indicating that the macrophage is an iron-limiting environment (16). Based on these observations, it is tempting to postulate that iron deprivation during macrophage infection can be a signal for induction of *icl* and possibly other genes required for the persistence of *M. tuberculosis*.

Examining the promoter regions of the group of iron-regulated IdeR-independent genes did not reveal obvious conserved sequences. Since the sequences recognized by other putative iron regulators, such as FurA/B and SirA, are unknown, it is not possible to predict whether these genes would be regulated by one of these proteins. Future studies should address the mechanisms for their iron-dependent control.

IdeR was found to be necessary for *M. tuberculosis* to respond effectively to oxidative stress. Since expression of *katG* or *sodA* was not affected by the *ideR* mutation, we believe that the requirement for IdeR is indirect. It is possible that in the absence of IdeR, the amount of redox-reactive iron is enhanced. Further studies are required to understand how IdeR specifically contributes to oxidative-stress defense in this mycobacterium.

In this study, we have extended our knowledge of the role of IdeR in *M. tuberculosis* and the response of this mycobacterium to iron levels. Our results indicate that IdeR is an essential regulator with a major role in controlling iron metabolism through its roles as a repressor of siderophore production and as a positive modulator of iron storage. The essential nature of IdeR makes it a potential candidate for chemotherapy, although as shown in this study, mutations that overcome the lethal effect of inactivating IdeR might arise under certain conditions. Finally, investigating the role of IdeR-regulated genes in iron acquisition will allow a better understanding of the mechanisms used by *M. tuberculosis* to survive low-iron environments encountered during infection and should provide additional potential targets for therapeutic intervention.

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