Tubercle and Lung Disease (1998) **79**(2), 99–106
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tuld.1998.0011

Protective role of the *Mycobacterium smegmatis IdeR* against reactive oxygen species and isoniazid toxicity

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Summary *Objective*: To understand the mechanism by which IdeR is necessary for maintaining wild type levels of KatG and SodA enzyme activity and normal isoniazid (INH) resistance.
Design: To identify the step(s) of SodA and KatG function that were affected by the *ideR* mutation, quantitative western immunoassays and ribonucleic acid (RNA) hybridizations were performed. To see if the increased INH sensitivity of the *ideR* mutant was caused by lower SodA activity, the *Mycobacterium smegmatis* sod gene was inactivated.
Results: The levels of KatG and SodA mRNA and protein in the *M. smegmatis IdeR* mutant are decreased to approximately 20–40% of those observed in the wild type parent strain. This is quantitatively similar to the decrease in KatG and SodA enzyme activities originally observed in the *ideR* strain. The *M. smegmatis sodA* mutant was slightly more sensitive to INH, compared to the wild type strain and was more resistant than the *ideR* mutant.
Conclusion: IdeR is necessary for full expression of the *M. smegmatis katG* and *sodA* genes. It is not yet known whether this protein acts directly at the gene level. The lower levels of SodA contribute slightly to the increased susceptibility to INH of the *ideR* mutant, but cannot explain the magnitude of the INH sensitivity observed when IdeR is not present. These data suggest that IdeR is a regulator of the cellular stress response, as it has a protective role in cells facing environmental stresses, such as increased levels of reactive oxygen species and INH toxic intermediates. These conclusions do not necessarily apply to IdeR's role in *M. tuberculosis* physiology, since we have not inactivated its gene in this pathogen.

INTRODUCTION

Mycobacteria are the leading cause of human mortality worldwide by infectious agents. Among the diseases caused by mycobacteria, tuberculosis and leprosy remain major health problems that affect tens of millions of individuals per year. To develop new therapeutics and more effective vaccines, a better understanding of mycobacterial physiology, pathogenesis and host interaction is needed.

Since bacterial pathogens encounter different environments during the dynamic process of pathogenesis, adaptive responses and modulation of virulence gene expression are required for their survival.[1] Response to oxidative stress is crucial for the survival of aerobic pathogens. Reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen can be generated as by-products of oxygen metabolism during respiration.[2] Oxidative stress occurs when abnormally high levels of ROS are generated, e.g. by the oxidative burst of phagocytes,[3] exposure to radiation[4] or redox drug.[5] ROS can damage all major biological molecules, lipids, proteins, and deoxyribonucleic acid (DNA),[6] and are important to the host's microbicidal activity. In response, pathogens have evolved mechanisms to defend themselves against both endogenous and exogenous oxidative damages. ROS detoxification enzymes, superoxide dismutase (SOD) and catalases have been shown to contribute to protection and/or may

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Received: 12 June 1998; Revised: 14 August 1998; Accepted: 21 August 1998

play a role in virulence of pathogens such as *Shigella flexneri*,[7] *Campylobacter jejuni*,[8] *Bordetella pertussis*,[9] *Salmonella typhimurium*,[10] *Yersinia enterocolitica*,[11] *Haemophilus influenzae*,[12] and *Neisseria meningitidis*.[13] The catalase/peroxidase KatG of *Mycobacterium bovis* has been associated with virulence in guinea pigs.[4] Several other protective enzymes have been described and may play a role in mycobacterial survival and pathogenesis in humans.[15–17]

Regulation of bacterial responses to oxidative stress has been most extensively studied in enterobacteria.[18] However, oxidative stress response of mycobacteria may not follow the pattern of these model organisms. *M. smegmatis* pretreated with low doses of hydrogen peroxide was resistant to killing by lethal doses of hydrogen peroxide.[17] This protective response to hydrogen peroxide is analogous to the OxyR response found in Gram negative bacteria, but no OxyR homolog has been described in *M. smegmatis* so far. In contrast, *M. avium*, *M. bovis* bacille Calmette Guérin and *M. tuberculosis* did not exhibit any protection.[17] A putative OxyR homolog has been found in *M. avium* and *M. leprae* that appears to be intact, whereas in strains of the *M. tuberculosis* complex the *oxyR* homolog contains deletions, frameshifts and nonsense mutations resulting in its inactivation.[17,19] It has been hypothesized that pathogenic mycobacteria may be continuously exposed to ROS and therefore express a constitutive defense to oxidative stress that would not require a functional OxyR.[17]

We have previously shown that the *M. smegmatis* IdeR plays a role in oxidative stress response and iron metabolism.[20] As a first step towards elucidation of the regulatory network controlling ROS detoxification enzymes in mycobacteria, we now demonstrate that inactivation of *ideR* in *M. smegmatis* results in decreased levels of KatG and SodA proteins and mRNAs. We also show that an *ideR* mutant of *M. smegmatis* is more sensitive to the front line antimycobacterial agent isoniazid (INH) and discuss possible explanations for this phenotype.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

M. smegmatis mc^{2}155[21] was the parent for the strains used in this study. Mycobacterial strains were grown on Middlebrook 7H10 solid medium (Difco) supplemented with 0.2% glycerol and 0.05% Tween 80 (7H10GT) or in Middelbrook 7H9 broth (Difco) supplemented with 0.2% glycerol and 0.05% Tween 80 (7H9GT) at 37°C. Kanamycin, when required, was added at a final concentration of 20 μg.ml^{-1}. *E. coli* was routinely grown in LB medium (Difco) at 37°C.

RNA isolation and slot-blotting

Total RNA was extracted from mycobacterial cultures grown exponentially in 7H9GT as previously described.[22] The RNA was further purified by DNase I treatment and extraction with phenol/chloroform/isoamyl alcohol 25:24:1 (v/v/v). The purified RNA was precipitated with ethanol and quantified by spectrophotometry. Slot-blots were performed using a Hybond N+ nylon membrane (Amersham) and the Bio-Dot SF kit (BioRad) according to the manufacturer's instructions. Blots were prehybridized for 15 min at 65°C in Rapid-Hyb buffer (Amersham). After adding radiolabelled probes, hybridization was carried out for 2 h at 65°C. Blots were washed three times at room temperature with 2X SSC, 0.1% SDS for 5 min, once with 1X SSC, 0.1% SDS for 15 min at 65°C and once with 0.1X SSC, 0.1% SDS for 15 min at 65°C. Radioactive signals were detected by autoradiography using Biomax-MR film (Kodak) and quantified by densitometry using a storage phosphor screen (Molecular Dynamics) and the Image Quant 1.11 software (Molecular Dynamics).

Construction of a *sodA* mutant of *M. smegmatis*

SOD5′2 (GTGGCYGAATACACYYTGCC) and SOD3′2 (TCAGYCGAAKATCARBCC) consensus primers derived from the sequences of *M. tuberculosis*, *M. leprae* and *M. avium* SOD gene extremities were used to amplify the *M. smegmatis sodA* gene by PCR. The PCR product was cloned and sequenced confirming that it was the *M. smegmatis sodA*. Based on the *M. smegmatis sodA* sequence, the forward primer SOD5′3 (5′-CAGATCAACGAGCTGCACC-3′) and the reverse primer SOD3′6 (5′-TTCTTCCGCCA-GATCGAGTGG-3′) were used to amplify by PCR a 180-bp internal fragment from the *sodA* gene from *M. smegmatis* mc^{2}155 chromosomal DNA. The 180-bp fragment was cloned into pCR2.1 (Invitrogen), creating pSM299. Plasmid DNA purified by using the Qiagen plasmid kit (Qiagen Inc.) was electroporated into *M. smegmatis* mc^{2}155 as previously described.[23] Transformants resistant to kanamycin were selected and *sodA* mutants were screened directly by assaying their SOD activity on gels.[24] Chromosomal DNA and whole-cell protein extract from colonies showing decreased SOD activity were used to confirm *sodA* disruption and decreased levels of SodA by Southern blot and Western blot analyses respectively. One of these recombinants was designated SM112.

Preparation of cell extracts and Western blotting

Mid-log mycobacterial cultures were grown in 7H9GT at 37°C. Crude protein extracts were obtained by sonication as previously described.[20] Western blotting was performed by standard procedures.[25] Briefly, proteins were transferred after denaturing polyacrylamide gel electrophoresis onto

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Immobilon-P membranes (Millipore) by semi-dry electroblotting for 45 min at 15V (Transblot SD, BioRad). Blots were blocked overnight with 5% (w/v) Blotto, 0.02% sodium azide at room temperature. Blots were incubated at room temperature for 2 h with rabbit polyclonal antibodies diluted at 1:1000 in Blotto. After washing with Blotto, blots were incubated at room temperature for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG goat antibodies (Sigma) at 1:20 000 diluted in Blotto. After washing twice with Blotto and once with PBS, alkaline phosphatase activity was visualized using BCIP/NBT phosphatase substrate (Kirkegaard & Perry Laboratories). Alternatively, the ECL Western blotting system (Amersham) was used according to the manufacturer's instructions. Signals were quantified by densitometry using the ScanAnalysis software (Biosoft).

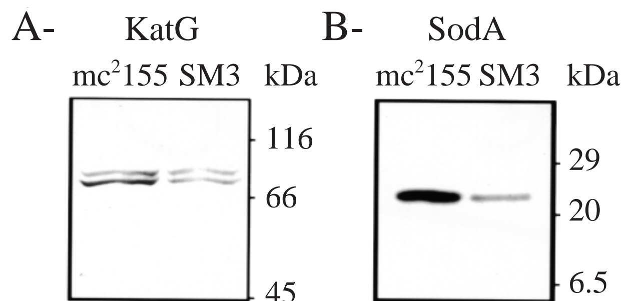

Fig. 1 Immunodetection of KatG and SodA in the *M. smegmatis* mc²155 wild-type strain and the *ideR*-defective mutant strain SM3. Western blot analysis was performed with whole-cell extracts of *M. smegmatis* mc²155 and *ideR*-defective mutant SM3. Fifty micrograms of total protein from each cell extract were separated by polyacrylamide gel electrophoresis and immunoblotted with anti-*M. tuberculosis* KatG (A) or anti-*M. tuberculosis* SodA (B) polyclonal antibodies.

Determination of growth inhibition by agar dilution assay and zone diffusion assay

Mycobacterial strains were grown to exponential phase and identical optical density in 7H9GT at 37°C. For agar dilution assays, 100 μl aliquots were diluted in fresh medium and spread on 7H10GT plates containing increasing concentrations of INH. After incubation for 5 days at 37°C, the percentage of survival was determined by multiplicating the number of colonies on the plates with INH by 100 and dividing by the number of colonies on the plates without INH. For zone diffusion assays, 10^6 bacteria were spread on 7H10GT plates. Paper disks (6.5 mm in diameter) (Schleicher Schuell) containing 10 μl of the inhibitory reagent tested were placed on top of the agar. The diameters of the zones of growth inhibition were measured after 3–5 days of incubation at 37°C.

RESULTS

An *ideR* mutant of *M. smegmatis* has reduced levels of catalase-peroxidase and SOD

We have previously shown that the inactivation of *ideR* in *M. smegmatis* results in a reduction of catalase/peroxidase and SOD activities.[20] In order to elucidate the mechanism by which *IdeR* exerts these effects, we first measured levels of KatG and SodA proteins in both mc²155 wild-type and SM3 *ideR* mutant strains. Whole-cell protein extracts from *M. smegmatis* mc²155 and SM3 were prepared and analyzed by Western blotting using polyclonal antibodies raised against *M. tuberculosis* catalase/peroxidase and SOD. A protein of approximately 80 kDa corresponding to the *M. smegmatis* KatG was observed in cell extracts of both wild-type and mutant strains. However, the amount of KatG found in SM3 was decreased by 78% compared to the wild-type (Fig. 1A). Similarly, a protein of apparent molecular weight of 23 kDa corresponding to the *M. smegmatis* SodA was observed in both strains but the SodA levels were reduced by 73% in the mutant strain SM3 (Fig. 1B).

M. smegmatis ideR positively controls the levels of *katG* and *sodA* mRNA

To determine whether the altered levels of KatG and SodA proteins were the result of reduced levels of their messenger RNAs, specific probes were used to measure the relative amounts of *katG* and *sodA* mRNAs in *M. smegmatis* wild-type and *ideR*-defective mutant strains (Fig. 2A). *katG* RNA levels were reduced by 79% and *sodA* RNA levels were reduced by 61% in mutant SM3 compared to the wild-type RNA levels (Fig. 2B). The above results indicate that the lowered SodA and KatG activities originally observed in ideR mutants[20] result from defects in gene expression, not enzyme function.

Inactivation of *ideR* increases *M. smegmatis* sensitivity to INH

Since the loss of KatG activity is associated with INH resistance in mycobacteria,[26] the INH sensitivity of wild-type and SM3 strains was compared (Fig. 3). Surprisingly, the *ideR* mutant strain showed increased sensitivity to INH compared to the wild-type strain. The MIC_{50} were 0.7 and 1.9 μg/ml for the SM3 and wild-type strains respectively. Thus, the decrease of KatG observed at the level of mRNA, protein and activity does not mediate resistance to INH in an ideR mutant strain as was found in *katG*-defective strains.[27,28]

SOD deficiency contributes slightly to the increased sensitivity to INH of an *ideR* mutant of *M. smegmatis*

As reactive oxygen species generated as byproducts of

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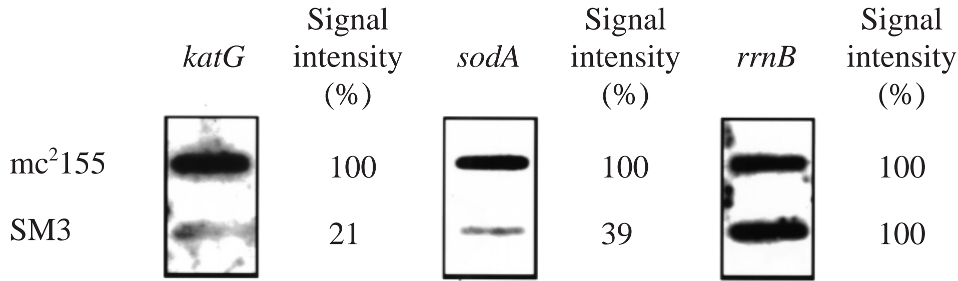

Fig. 2 Slot-blot analysis of RNA transcripts of the *M. smegmatis* mc^{2}155 wild-type strain and the *ideR*-defective mutant strain SM3. A. One microgram of purified RNA from the two strains was analyzed by slot-blotting with ^{32}P-labelled probes corresponding to the *M. smegmatis katG* and *sodA* genes. A probe corresponding to the *M. smegmatis rrnB* was used as a control. B. Amount of *katG* and *sodA* transcripts in the mutant strain relatively to the wild-type strain. The radioactive signals from A. were quantified by densitometry. Each amount was first normalized using the *rrnB* transcripts and the ratio of mutant-to-wild-type transcripts was then determined.

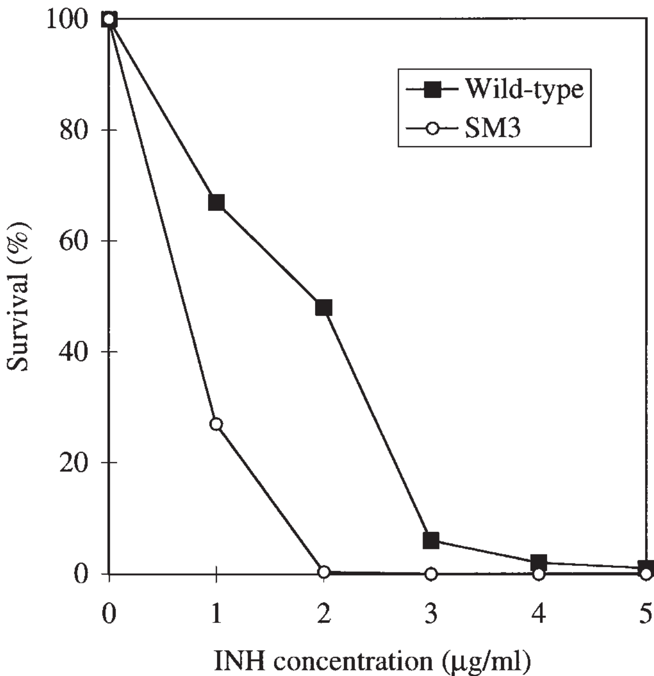

Fig. 3 Sensitivity of an *ideR*-defective mutant to INH. Mid-log mycobacterial cultures were grown in 7H9GT and ≈10^7 bacteria were diluted and plated on 7H10GT supplemented with various concentrations of INH.

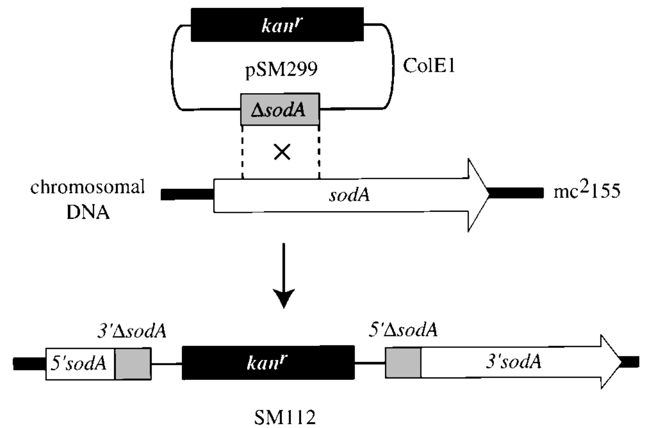

Fig. 4 Strategy of *sodA* disruption. A 180-bp internal fragment of the *M. smegmatis sodA* (Δ*sodA*) was cloned into the suicide plasmid pSM299, containing the ColE1 origin of replication which functions only in *E. coli* and a kanamycin resistance gene (*kan^r*). mc^{2}155 *M. smegmatis* was transformed with pSM299 and integration of the plasmid confered a kanamycin resistant phenotype. Homologous recombination between the 180-bp fragment on pSM299 and the chromosomal *sodA* gene led to disruption of the endogenous gene.

the INH activation process may contribute to INH toxicity,[29–30] the role of the SodA levels in the increased sensitivity of the *ideR* mutant to INH was investigated. The suicide plasmid pSM299 was used to create a *sodA* mutant strain of *M. smegmatis*, SM112, by single cross-over (Fig. 4). Cell extracts of both wild-type and SM112 strains were analyzed by Western blotting showing that SodA levels were greatly reduced in SM112 (data not shown). SOD activity gel electrophoresis showed that the activity band corresponding to SodA was absent (Fig. 5). However, the SM112 lysate contained a SOD activity that migrated slightly slower than the wild-type SodA (Fig. 5). The same activity band was observed with several different Campbell type disruptions of *sodA*, suggesting the presence of another SOD. It is possible that inactivation of SodA causes overexpression of this other SOD, as a similar compensatory phenomenon has been observed in a *Streptomyces lividans sodF* mutant.[31] In addition to the iron-dependent SodA, an open reading frame with similarity to Cu/ZnSODs is found in the Sanger *M. tuberculosis* H37Rv genomic database (MTCY22G10.29). It is possible that the other SOD observed in our experiments is related to the second ORF found in *M. tuberculosis.* However, such a protein has not been characterized yet in *M. smegmatis.*

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Fig. 5 SOD activity in the *M. smegmatis* mc^{2}155 wild-type strain, the *ideR*-defective mutant strain SM3 and the *sodA* mutant strain SMI 12. Whole-cell extracts of *M. smegmatis* mc^{2}155, SM3, and SM112 strains were submitted to native polyacrylamide gel electrophoresis and the gel was stained for SOD activity. Each lane contained 50 μg of total protein.

Table 1 Growth inhibition zones by INH in the *M. smegmatis* mc^{2}155 wild-type strain, the *ideR*-defective mutant strain SM3 and the *sodA* mutant strain SM112. Zone diffusion assays were performed using discs containing 10 μl of 10 mg/ml INH

Strains	INH[a] zone of inhibition, diameter (mm)[b]
mc^{2}155	20.2 ± 0.4
SM3	40.2 ± 0.4
SM112	25.4 ± 0.5

[a]10 mg/ml isoniazid.
[b]Experiments were conducted at least five times.

The sensitivity to INH of wild-type and SM112 strains was then compared by zone diffusion assay (Table 1). SM112 was slightly more sensitive to INH than the wild-type strain but more resistant than SM3 (Table 1), eventhough the level of SOD activity in SM112 was lower than the SM3 levels (Fig. 5). Therefore, the decreased SOD level only accounts for a small part of the increased susceptibility to INH of the *ideR* mutant strain.

An *ideR* mutant of *M. smegmatis* has wild-type levels of alkyl hydroperoxide reductase subunit AhpC

Since an *ahpC* mutant of *M. smegmatis* has been shown to be hypersensitive to INH,[32] the hypothesis that lower levels of AhpC could account for the SodA-independent susceptibility of the *ideR* mutant to INH was tested. Whole-cell protein extracts from *M. smegmatis* mc^{2}155 and SM3 were prepared and analyzed by Western blotting using polyclonal antibodies raised against *M. tuberculosis* AhpC (Fig. 6). A protein of approximately 25 kDa corresponding to the *M. smegmatis* AhpC was observed in comparable amount in both cell extracts.

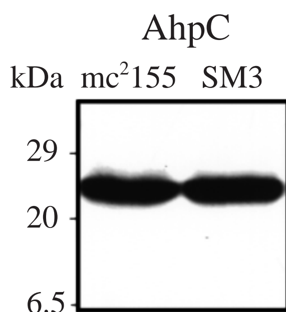

Fig. 6 Immunodetection of AhpC in the *M. smegmatis* mc^{2}155 wild-type strain and the *ideR*-defective mutant strain SM3. Western blot analysis was performed with whole-cell extracts of *M. smegmatis* mc^{2}155 and *ideR*-defective mutant SM3. 50 μg of total protein from each cell extract were immunoblotted with anti-*M. tuberculosis* AhpC polyclonal antibodies.

Hypersensitivity of an *ideR* mutant is not specific to INH

In order to determine whether the increased susceptibility of the *ideR* mutant strain was a result of a general oxidative stress hypersensitivity or a specific hypersensitivity to INH, the sensitivity of wild-type and SM3 strains to various inhibitory compounds was compared by zone diffusion assay (Table 2). The *ideR* mutant strain showed increased sensitivity to the oxidizing heavy metal cadmium and to the superoxide radicals generator plumbagin. However, the sensitivity to cumene hydroperoxide, rifampicin, and sodium dodecyl sulfate (SDS) of the *ideR* mutant was not significantly different from the wild-type.

DISCUSSION

We have previously demonstrated that IdeR acts as an iron-responsive repressor.[33] When complexed to Fe(II), IdeR binds to a specific palindromic consensus sequence in the promoter (IdeR box) and inhibits transcription of iron-regulated genes in *M. smegmatis*,[34] and *M. tuberculosis*.[35] We have also previously demonstrated that inactivation of *M. smegmatis ideR* causes a decrease in catalase/peroxidase and SOD activities.[20] In this study, we further investigate the role of the mycobacterial IdeR in the regulation of the oxidative stress response. Here, we show that an *ideR* mutant of *M. smegmatis* has reduced levels of KatG, SodA proteins and their respective transcripts. These decreases are quantitatively very similar to those observed in the KatG and SodA activities of the same

Table 2 Growth inhibition zones by various stress reagents in the *M. smegmatis* mc^{2}155 wild-type strain and the *ideR*-defective mutant strain SM3. Zone diffusion assays were performed using discs containing 10 μl of cadmium chloride, cumene hydroperoxide, plumbagine, rifampicin, or sodium dodecyl sulfate at various concentrations

	Zone of inhibition, diameter (mm)[a]				
Strains	CAD	CHP	PBG	RIF	SDS[b]
mc^{2}155	22.5 ± 4.9	37.5 ± 0.5	35.8 ± 0.4	24.0 ± 0.9	22.0 ± 0.0
SM3	33.0 ± 5.0	39.7 ± 1.3	52.6 ± 0.5	25.0 ± 0.9	22.0 ± 0.0

[a]Experiments were conducted three to five times.
[b]CAD: 100 mM cadmium chloride; CHP: 200 mM cumene hydroperoxide; PBG: 10 mM plumbagine; RIF: 10 mg/ml rifampicin; SDS: 20% sodium dodecyl sulfate.

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mutants. This indicates that the effect of the *ideR* mutation is at the level of gene expression and that IdeR positively regulates *katG* and *sodA*. The molecular mechanism of this regulation remains to be elucidated. It is important to stress that the oxidative stress responses of *M. tuberculosis* are different from those of *M. smegmatis*[17,32] and that our observations may not apply to this pathogen.

Very little is known about the regulation of KatG in mycobacteria. *M. smegmatis* has an adaptive response to hydrogen peroxide analogous to the OxyR response from Gram negative bacteria.[17] However, OxyR has not yet been described in *M. smegmatis.* A putative OxyR has been reported in *M. avium*, *M. leprae* and as a pseudogene in strains of the *M. tuberculosis* complex.[17,19] Like OxyR in *Escherichia coli* and *S. typhimurium*,[36,37] IdeR controls positively KatG, yet is not a functional homolog of OxyR. *ahpC*, which is activated by OxyR in *E. coli*, is not activated by IdeR in *M. smegmatis* as the *ideR* mutant and the wild-type strains showed similar levels of AhpC, and OxyR of *E. coli* has not been reported to repress iron uptake genes. Interestingly, it has recently been shown that KatG can be induced by an OxyR-independent mechanism in *E. coli*.[38,39] Although the nature of this mechanism remains unclear, σ^s (also designated RpoS, KatF, Nur, AppR, Csi-2 and σ^{38}) and Fur have been proposed as putative positive regulators of KatG in addition to OxyR, as both *rpoS* and *fur* mutants have reduced levels of *katG* transcription and activity.[39,40] Supporting the hypothesis of Fur as a positive regulator of catalase, *P. aeruginosa fur* mutants have also been shown to have decreased total catalase activity.[41] IdeR and Fur are both iron-dependent repressors and they may be functional homologs with respect to catalase regulation as well.

This is the first report on regulation of SodA in mycobacteria. Homologs of SoxR and SoxS, the two activators of SodA in *E. coli*, have yet to be reported. Both Fur and IdeR are central components in iron metabolism and the oxidative stress response, and their inactivation leads to derepression of iron uptake and to oxidative stress.[20,42] Fur has been shown to repress s*odA* in *E. coli*[43,44] and IdeR appears to positively control SodA in mycobacteria. Therefore, these two analogous regulators show significant differences in their regulons. Since iron exacerbates ROS toxicity by catalyzing the formation of extremely reactive species such as hydroxyl radicals.[45,46] IdeR appears to have a protective role by switching off iron uptake while promoting oxidative defense in the mycobacterial cell. Paradoxically, under conditions that require protection against oxidative stress, *E. coli* Fur represses *sodA.* It has been hypothesized that in a low iron environment, the iron uptake machinery is derepressed and could lead to a transient iron overload before Fur could repress again. Thus, SodA could protect against this transient oxidative burst.[42,47] However, this hypothesis remains to be proven rigorously. Attempts to decipher the connections of the regulatory networks are complicated because:

1. *sodA* is under complex regulation involving six regulators (SoxRS, SoxQ, Fur, ArcA, IHF and Fnr)[48]
2. *sodB*, which codes for the iron SOD, seems to be positively regulated by Fur[43]
3. the oxidative stress response overlaps with other stress responses.[49]

Two putative homologs of *fur, furA* and *furB*, have been recently found in mycobacteria.[50,51] It is not known if the mycobacterial *fur* genes are expressed and thus it remains to be proven that FurA and FurB are functional homologs of Fur. Since inactivation of IdeR results in only a partial derepression of siderophore synthesis,[20] one can speculate that FurA and/or FurB constitutes the hypothesized second repressor. Interestingly, *furA* is found directly upstream of *katG* in mycobacteria,[50] suggesting a possible role for this protein in *katG* regulation.

IdeR could regulate *katG* and *sodA* transcription or affect mRNA stability and experiments are in progress to test these possibilities. Preliminary gel shift assays using DNA fragments covering 1 kb upstream of the *M. tuberculosis katG* and *sodA* genes did not show direct binding of IdeR (Dussurget & Smith, unpublished data). Therefore, it suggests that IdeR may control *katG* and *sodA* transcription indirectly. However, a direct effect of IdeR cannot be eliminated since the mechanism of regulation may be different in *M. smegmatis* and *M. tuberculosis.* Since the upstream sequence of the *M. smegmatis katG* and *sodA* was not available, this hypothesis has not been tested yet. It is also possible that an essential cofactor or signal necessary for IdeR binding was missing in our assay.

We also found that inactivation of IdeR led to an increased sensitivity to INH. Since the loss of KatG has long been known to be associated with INH resistance in mycobacteria,[26,27] it was somewhat surprising that a strain producing low levels of KatG was also more sensitive to INH. It has been proposed that KatG participates in the activation of INH that inhibits InhA or another enzyme involved in mycolic acid biosynthesis and possibly other targets.[51–53] However, multiple mechanisms of action of INH have been postulated because enteric bacteria lacking mycolic acids can be made susceptible to INH.[54] Both *oxyR* mutants and *ahpC-katG* double mutants are more susceptible to INH in *E coli* and *S. typhimurium*.[54,55] Free radical species and ROS are generated during the INH activation process, which could also lead to INH toxicity.[29,30,56–58] Plumbagin-treated *M. smegmatis* is more susceptible to INH, an effect that can be reversed by over-expressing the SOD.[59] We hypothesized that the low levels of KatG in the *ideR* mutant are sufficient for INH activation and that the levels of SodA are reduced enough to increase the sensitivity to INH.

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To test this hypothesis, we constructed a *sodA* mutant of *M. smegmatis.* The mutant had SOD activity levels that were lower than those of the *ideR* mutant strain and this SOD defect resulted in a slight increase in INH susceptibility. This confirms that superoxide anion radicals play a role in INH toxicity in *M. smegmatis* but suggests that their role in the *ideR* mutant hypersusceptibility to INH is minor. Besides, ROS other than superoxide radicals may have deleterious effects on the cells. This could explain why AhpC mutants in *M. smegmatis* are more susceptible to INH.[32] However, organic peroxides are not likely to contribute to the INH hypersusceptibility of the ideR mutant since it produced wild-type levels of AhpC and was not more sensitive to cumene hydroperoxide than the wild-type strain.

In view of the data discussed above and the response of the *ideR* mutant to various stress conditions, i.e. oxidizing agents, antibiotics and SDS, it appears that inactivation of IdeR causes a hypersensitivity to oxidative stress and yet does not mediate a global stress hypersensitivity, nor a selective INH hypersensitivity. However, the response of the *ideR* mutant to oxidative stress was limited, since it did not show increased sensitivity to cumene hydroperoxide. Similarly, we found no change in sensitivity to rifampicin, which suggested that the INH sensitivity of the *ideR* mutant was not a generalized antibiotic sensitivity.

IdeR is the first pleiotropic regulator playing a protective role in response to different cellular stresses described in mycobacteria. Based on these results and previous data,[20] we propose that the inactivation of *ideR*, which (1) decreases the synthesis of enzymes such as KatG and SodA that are capable of reducing oxidants and (2) deregulates iron uptake, could lead to hypersensitivity to some ROS and to INH intermediates. The relevance of these observations to *M. tuberculosis* awaits the isolation of an *ideR* mutant in this pathogenic species.

ACKNOWLEDGMENTS

We thank Carl Nathan, Clifton Barry III, Valerie Snewin and Douglas Young for anti-AhpC, anti-KatG, and anti-SodA antibodies, respectively. We also acknowledge the scientific participation of Cesar Perez and Yan Makeyev in some aspects of this project, and valuable discussion with Manuel Gomez, Jeanie Dubnau, Ben Gold, Riccardo Manganelli and Juliano Timm.

This work was supported by grant GM3265 I from the National Institutes of Health to IS, and by a fellowship from the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche to OD.

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