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Compensatory *ahpC* Gene Expression in Isoniazid-Resistant *Mycobacterium tuberculosis*

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Mutations that eliminate KatG catalase–peroxidase activity prevent activation of isoniazid and are a major mechanism of resistance to this principal drug for the treatment of *Mycobacterium tuberculosis* infections. However, the loss of KatG activity in clinical isolates seemed paradoxical because KatG is considered an important factor for the survival of the organism. Expression of either KatG or the recently identified alkyl hydroperoxidase AhpC was sufficient to protect bacilli against the toxic effects of organic peroxides. To survive during infection, isoniazid-resistant KatG mutants have apparently compensated for the loss of KatG catalase–peroxidase activity by a second mutation, resulting in hyperexpression of AhpC.

As an intracellular pathogen residing within macrophages, *Mycobacterium tuberculosis* (MTB) is well equipped to resist toxic oxygen species. However, a principal drug used to treat tuberculosis, isoniazid (isonicotinic acid hydrazide, or INH) interacts with components of the mycobacterial defense against oxidative stress in complex ways. INH is a prodrug that requires activation to an unstable electrophilic species by the catalase-peroxidase KatG, with hydrogen peroxide (H_2O_2) acting as an electron sink for the reaction (*1–3*). Once activated, INH inhibits the biosynthesis of cell wall mycolic acids (*4*), ultimately compromising the inert and largely impenetrable barrier that protects mycobacteria against reactive oxygen species and other environmental insults (*5*). KatG is the only MTB enzyme capable of activating this drug. As a result, KatG-mutant MTB strains are INH resistant.

Tuberculosis bacteria appear to rely on the constitutive defense afforded by their cyclopropanated cell wall mycolic acids, having virtually eliminated an inducible oxidative stress response from their genetic repertoire (*6*). In other bacteria, a peroxide-inducible genetic response mediated by the transcription factor OxyR is the primary defense against oxidative stress (*7, 8*). However, the recently identified *oxyR* gene of MTB is vestigial, containing numerous frameshifts and deletions (*6, 9*). Without OxyR, the only MTB protein whose expression is peroxide-inducible is KatG, and this induction is insufficient to protect against H_2O_2 challenge (*6*). It therefore seems paradoxical that loss of KatG function is the major means by which the tubercle bacilli acquire resistance to isoniazid (*10, 11*). It is unclear how these bacteria adapt to loss of KatG, their only catalase-peroxidase and their only peroxide-responsive gene product, when this activity is considered an important component of intracellular survival (*12*).

To evaluate the interaction of INH and H_2O_2 in TB-complex bacilli, we simultaneously administered subinhibitory concentrations of both H_2O_2 and isoniazid to *Mycobacterium bovis* BCG (an avirulent member of the TB complex). As in Gram-negative bacteria and the soil saprophyte *Mycobacterium smegmatis* (*13*), synergy was readily apparent. A reduction in cell viability by a factor of 100 was noted after 72 hours exposure to concentrations of H_2O_2 and INH that separately had no effect in this assay (Fig. 1). When subinhibitory concentrations of both agents were supplied to a *katG*-deleted BCG strain, no synergy was observed (*14*). The observed synergy is consistent

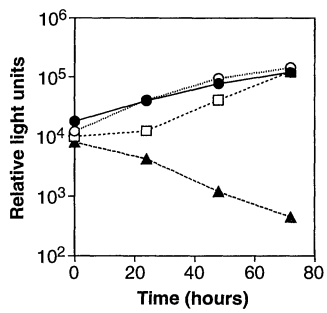

Fig. 1. Synergistic interaction of H_2O_2 and isoniazid. *M. bovis* BCG Connaught (ATCC 35745) transfected with an integrating plasmid constitutively expressing the firefly luciferase (*lux*) gene product (BCG:r361lux) (*23*) was grown at 37°C in 7H9 media supplemented with albumin and dextrose, but in the absence of exogenous catalase (7H9 no cat). Cells were passaged twice at low density [absorbance at 540 nm (A_{540}) ≤ 0.01] before dilution to ~1×10^5/ml for treatment. Cell viability was determined by monitoring light expression as described (*23*). (●) No H_2O_2, no INH; (○) no H_2O_2, 0.06 μg/ml INH; (□) 130 μM H_2O_2, no INH; and (▲) 130 μM H_2O_2, 0.06 μg/ml INH.

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with the suggestion that increasing the oxidative burden on MTB in vivo should improve the efficacy of isoniazid (*13*).

H_2O_2 exposures that result in synergy with INH induce expression of MTB KatG, even in the absence of a functional OxyR (6). This induction is due to activation of the *kat*G promoter. We cloned the *kat*G promoter region upstream of the firefly luciferase (*lux*) gene and monitored expression of this construct in BCG (*15*). *Kat*G-*lux* expression increased seven-fold in this strain after H_2O_2 treatment, consistent with the level of induction observed previously at both the transcriptional and translational levels. Because peroxide exposures sufficient to induce KatG do not protect MTB from a lethal H_2O_2 dose as they do in *Escherichia coli*, *Salmonella typhimurium*, or *M. smegmatis* (6, 8, *13*), the relevance of the inducible KatG response to MTB survival was unclear. We therefore examined the effect of KatG induction on sensitivity to organic hydroperoxides. Induction of KatG with H_2O_2 enhanced the survival of BCG exposed to cumene hydroperoxide by about 35-fold (Fig. 2). A BCG strain deleted for *kat*G was much more sensitive to oxidative stress, and treatment of this strain with subinhibitory levels of H_2O_2 did not enhance protection against either peroxide species (*14*). These data suggest that KatG may function in vivo to protect against organic hydroperoxides.

The observation that KatG(-) MTB are more sensitive to organic peroxides may explain the attenuated virulence of these strains. Previous analysis suggested that MTB may compensate for loss of KatG function with other changes in protein expression (6). We therefore examined INH-resistant, KatG-mutant MTB strains, including clinical isolates, for common changes in protein expression profiles. All eight mutant strains examined expressed a 22-kD protein with an isoelectric point (pI) of ~4 at substantially higher levels than INH-sensitive strain H37Rv (Fig. 3). NH_2-terminal sequence analysis identified this protein as the recently reported MTB AhpC, a putative alkyl hydroperoxidase (GenBank entry U18264) (6). The eight INH-resistant, KatG-negative strains overexpressed AhpC to a varying extent, whereas nine KatG wild-type isolates all produced the AhpC protein at the low wild-type levels. The strict correlation between loss of KatG function and overexpression of AhpC in MTB clinical isolates has also been noted recently by others (*16*). To test if AhpC overexpression preceded the loss of KatG activity, we selected for INH-resistant mutants on solid media (*17*). All nine KatG-deficient laboratory isolates showed no evidence of AhpC up-regulation (*14*). These strains differ from other KatG(-) laboratory or clinical isolates in that they have never experienced passage through animals or liquid culture. The compensatory mutations that up-regulate *ahp*C expression apparently require a second selection event during infection or further passage in culture.

The 5′ regions of confirmed *kat*G mutants shown to hyperexpress AhpC were sequenced to identify potential promoter mutations. Each AhpC–up-regulated isolate was shown to harbor at least one mutation in the region from 39 to 81 base pairs (bp) upstream of the *ahp*C start codon (Table 1). We demonstrated that these mutations could positively affect promoter activity by comparing the ability of the wild-type *ahp*C 5′ region and two analogous 5′ seg-

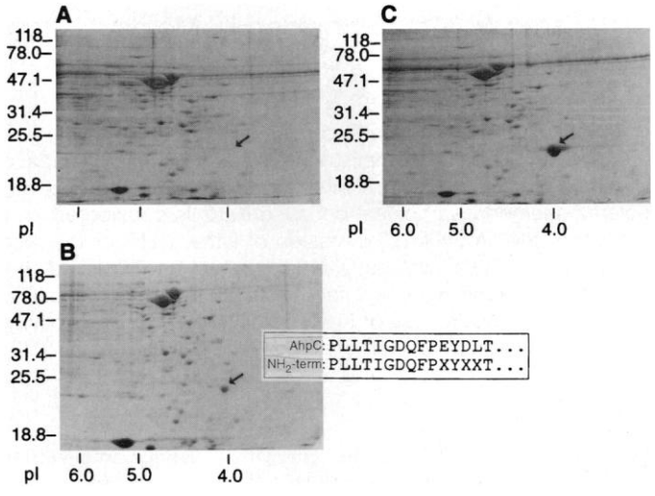

Fig. 3. Two-dimensional gel electrophoresis of KatG(-) isoniazid–resistant clinical isolates reveals up-regulation of AhpC. Isoelectric focusing was done in the first (horizontal) dimension, and SDS–polyacrylamide gel electrophoresis was done in the second (vertical) dimension. Molecular size standards are shown on the left (in kilodaltons), and isoelectric point (pI) standards are shown along the bottom. (**A**) Wild-type *M. tuberculosis* H37Rv. The arrow points to the spot where AhpC would be expected to appear. (**B**) A KatG(-) clinical isolate of *M. tuberculosis* ATCC35822. The arrow points to the up-regulated spot. (**Inset**) Microsequencing results from several combined spots aligned with the NH_2-terminal amino acid sequence of the AhpC protein from MTB. Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; L, Leu; P, Pro; Q, Gln; T, Thr; W, Trp; and Y, Tyr; X, any amino acid. (**C**) *M. tuberculosis* H37Rv:pMH91, in which a 1.3-kb Not I–Pst I fragment containing a 459-bp upstream sequence and the complete AhpC open reading frame (ORF) from MTB strain Erdman was inserted into an extrachromosomal shuttle vector and transformed into MTB strain H37Rv.

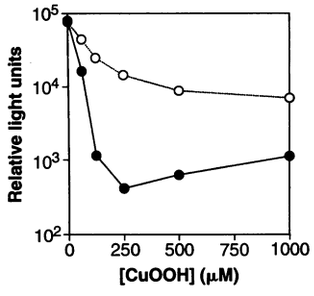

Fig. 2. Preinduction of KatG protects against a subsequent lethal challenge with cumene hydroperoxide. BCG:r361lux was grown as described (Fig. 1) and then diluted to ~1 × 10^6 colony-forming units/ml. Pretreated cells (○) were exposed to 100 μM H_2O_2 in 7H9 (no cat) at 37°C for 1 hour, followed by 4 hours of exposure to the cumene hydroperoxide concentrations shown. Control cells (●) were treated identically, except that pretreatment did not include any H_2O_2. Cell viability was determined by monitoring light output as described (*23*).

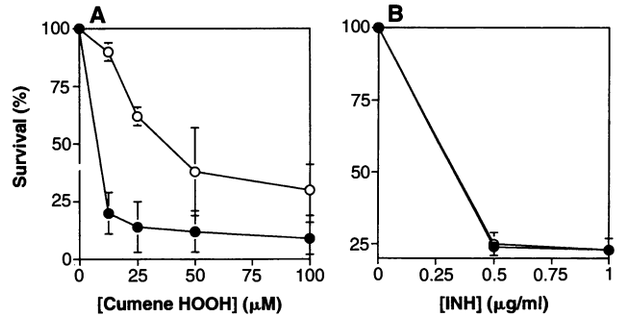

Fig. 4. Overexpression of AhpC protects wild-type *M. tuberculosis* against cumene hydroperoxide, but not against INH. The fidelity of each construct was confirmed by sequence analysis. (**A**) MTB H37Rv:pMV206H (vector control) (●) and MTB H37Rv:pMH91 (○) were grown to mid-log phase and then diluted to an A_{650} of 0.05, and cumene hydroperoxide was added to the indicated concentrations. Absorbances were measured daily for 1 week, and the percentage survival was calculated by comparing the absorbance of treated cultures with comparably diluted untreated cultures. (**B**) Symbols and procedures as in (A), but with INH added in place of cumene hydroperoxide.

Table 1. Promoter mutations upstream of *ahpC* contribute to AhpC up-regulation in KatG-deficient MTB. All of the constructs were generated by polymerase chain reaction of the MTB *ahpC* 5′ regions; they all include 551 bp upstream of the *ahpC* start codon plus the NH_2-terminal 16 amino acids of *ahpC* fused to the *lux* ORF. The fidelity of each construct was confirmed by sequence analysis. RLU, relative light units.

Strain	KatG	AhpC	Promoter	AhpC-lux (RLU)	Source
Erdman	+	−	CACCTTTGCCTGACAGCGACTTCACGGCACGATGGAATGTCGC	1.0	MTU18264
MBV35723	+	−	.....		MBU24083
MBV35729	−	+	.....T.....		MBU24084
MTB35822	−	+	.....T.....T	18	This study
MTBK10516	−	+	.....A.....	9	This study
MTB35825	−	+	.....A.....		This study
BCG35747	−	+	.....T.....		This study
MTB35835	−	+	.....T.....		This study
MTB35823	−	+	T.....		This study
MBV35727	−	+	.....C.....A.....		This study

ments from putative mutant promoter regions to drive expression of a *lux* reporter gene in BCG transformants. The wild-type *ahp*C 5′ region exhibited substantial promoter activity, but the putative promoter sequence of MTB35822, containing a single C to T substitution, had nearly 20 times the promoter activity of the wild-type sequence. Similarly, the same region of isolate K10516, with a single G to A conversion, showed nine times the wild-type promoter activity (Table 1).

AhpC is similar to a family of bacterial and eukaryotic antioxidant proteins with alkyl hydroperoxidase (Ahp) and thioredoxin-dependent peroxidase (TPx) activities (*18*, *19*). The recent identification of thioredoxin in MTB suggests that AhpC may play a similar role in this organism (*20*). To test the role of AhpC in the tubercle bacilli, we cloned *ahp*C from MTB strain Erdman into a multicopy plasmid vector and tested transformants (H37Rv:pMH91) for sensitivity to cumene hydroperoxide. In the context of the supercoiled multicopy plasmid vector pMH91, *ahp*C gene expression was even higher than that seen from the clinical INH-resistant KatG-mutant strains (Fig. 3C). The AhpC-overexpressing strain was significantly more resistant to cumene hydroperoxide than was the wild-type strain (Fig. 4A). Similar results were obtained in BCG where, in addition, the AhpC gene from clinical isolate K10516 (BCG:pMH94) was also tested. With weaker levels of overexpression, BCG:pMH94 was also more resistant to cumene hydroperoxide, albeit to a lesser extent (*14*). Thus, increased AhpC expression appears to compensate for the loss of KatG activity in the detoxification of organic peroxides.

Independent of its role in the detoxification of organic peroxides, increased AhpC expression could directly alter resistance to isoniazid. Presumed OxyR-inducible expression of *ahp*C in the INH-resistant *Mycobacterium avium* and *Mycobacterium leprae* have been invoked to support this theory (6, 9). To determine whether AhpC can directly detoxify isoniazid, we tested the INH sensitivity of BCG carrying the entire *oxy*R-*ahp*C region from *M. avium*. After direct plating or growth in liquid media containing INH, no significant differences in sensitivity to drug were observed. We then tested the INH sensitivity of H37Rv:pMH91, which massively overexpresses AhpC (Fig. 3C), and did not observe any increased survival in the presence of clinically relevant levels of isoniazid (Fig. 4B). In addition, *ahp*C loci from clinical isolates that hyperexpress the protein were tested in *M. smegmatis*, BCG, or H37Rv, on plates or in liquid media. Under no condition was the *ahp*C locus from a clinical isolate able to induce a measurable level of INH resistance in the absence of a *kat*G mutation (*14*). Furthermore, we have yet to observe AhpC hyperexpression in passaged INH-resistant strains of MTB, or in clinical isolates without KatG lesions. These data refute a direct role for AhpC in the detoxification of isoniazid.

Reactive oxygen species are continuously generated by the normal biochemical processes of an organism (*21*, *22*). Toxic oxygen intermediates of all types react readily with cellular components and would generate organic peroxide forms of mycobacterial lipids and proteins. The principal role of induced KatG may be to detoxify these cellular components damaged by oxidative stress. Clearly, exposure to isoniazid presents MTB with a dilemma. Although the tubercle bacilli readily develop resistance to INH by preventing its activation, they appear to sacrifice one important component of their survival machinery, KatG, to do so. In the absence of an inducible OxyR-type regulatory mechanism, we propose that compensatory *ahp*C promoter mutations are selected from KatG-deficient, INH-resistant MTB during infection in vivo to mitigate the added burden imposed by organic peroxides on these strains. Considered by many an old, unsophisticated antibiotic, INH may be more properly considered a prototype for new antimicrobials that interact with specific bacterial defense mechanisms, such as those targeting oxidative stress.

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15. A 675-bp sequence upstream of the *katG* gene of MTB strain Erdman was used to drive expression of the *lux* gene in an integrating shuttle vector. Fidelity of constructs was determined by sequence analysis. Transformations into BCG and *lux* assays were done as described (*23*).
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17. Mid-log H37Rv were diluted and spread on 7H11 plates with INH (0, 0.5, 1.0, and 10 μg/ml). INH-resistant single colony isolates were re-streaked at each level on fresh plates with the same amount of INH, and then bacteria were collected and lysed as described (*6*). Lysates were probed by protein immunoblot with polyclonal antisera to KatG, and KatG(-) lysates were screened by two-dimensional gel electrophoresis for the up-regulation of AhpC.
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