Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 13212–13216, November 1996
Microbiology

Molecular basis for the exquisite sensitivity of *Mycobacterium tuberculosis* to isoniazid

(oxidative stress/*ahpC*/*oxyR*/gene replacements/mycobacteria)

Y. ZHANG*, S. DHANDAYUTHAPANI*, AND V. DERETIC*†‡

*Department of Microbiology, University of Texas Health Sciences Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284-7758; and
†Department of Microbiology and Immunology, 5641 Medical Science Building II, University of Michigan Medical School, Ann Arbor, MI 48109-0620

Communicated by Irwin C. Gunsalus, University of Illinois, Urbana, IL, August 28, 1996 (received for review May 14, 1996)

ABSTRACT **The exceptional sensitivity of *Mycobacterium tuberculosis* to isonicotinic acid hydrazide (INH) lacks satisfactory definition. *M. tuberculosis* is a natural mutant in *oxyR*, a central regulator of peroxide stress response. The *ahpC* gene, which encodes a critical subunit of alkyl hydroperoxide reductase, is one of the targets usually controlled by *oxyR* in bacteria. Unlike in mycobacterial species less susceptible to INH, the expression of *ahpC* was below detection limits at the protein level in INH-sensitive *M. tuberculosis* and *Mycobacterium bovis* strains. In contrast, AhpC was detected in several series of isogenic INH-resistant (INH^r) derivatives. In a demonstration of the critical role of *ahpC* in sensitivity to INH, insertional inactivation of *ahpC* on the chromosome of *Mycobacterium smegmatis*, a species naturally insensitive to INH, dramatically increased its susceptibility to this compound. These findings suggest that AhpC counteracts the action of INH and that the levels of its expression may govern the intrinsic susceptibility of mycobacteria to this front-line antituberculosis drug.**

Tuberculosis has never ceased to be a global health problem (1). Much of the recent attention that this disease has received can be attributed to the resurgence of tuberculosis in industrialized countries. This is further compounded by the emergence of drug-resistant strains of the etiologic agent *Mycobacterium tuberculosis*, including variants recalcitrant to treatments with the front-line antituberculosis agent isonicotinic acid hydrazide (isoniazid; INH) (1). Since its introduction in 1952 (2) as a potent agent for treatment of tuberculosis, INH has proven to be an invaluable therapeutic agent. However, despite numerous proposals (3–5) and recent developments (6, 7), the effects of this compound on the mycobacterial cell still lack a complete definition.

The initial advances in understanding the antimycobacterial action of INH came from the explorations of the mechanisms of the emergence of INH resistance in *M. tuberculosis* (8). Based on recent genetic analyses, it has been widely accepted that *katG*, a gene encoding catalase peroxidase, is a major site of mutations conferring INH resistance on *M. tuberculosis* (9, 10). These analyses have led to the current model in which INH is activated by a peroxidatic reaction in the presence of KatG (3, 5, 11). The activated product of INH is believed to act on InhA (6, 7, 12), an enzyme implicated in mycolic acid synthesis (6). In addition to the proposed action of INH on InhA and other putative targets (5, 6), the intracellular metabolism of INH is known to generate reactive oxygen intermediates (11, 13–16) that may have deleterious effects on mycobacterial cells.

While the majority of current investigations have been aimed at understanding the emergence of INH-resistant (INH^r) strains, we have recently addressed the fundamental question concerning the mechanisms underlying the natural hypersensitivity of *M. tuberculosis* to INH (17, 18). This organism stands out among mycobacteria as being several orders of magnitude more sensitive to INH than the majority of other species (3, 5). In efforts to investigate this phenomenon, we have recently shown (17) that all strains of *M. tuberculosis* and other members of the *M. tuberculosis* complex (*Mycobacterium africanum*, *Mycobacterium bovis*, and *Mycobacterium microti*) are natural mutants in *oxyR*, the putative *M. tuberculosis* equivalent of the central regulator of peroxide stress response in enteric bacteria. Similar results have been reported by others (19). The sequence of the *oxyR* region and the lesions inactivating the *oxyR* gene are identical in all type strains of the *M. tuberculosis* complex, which also share high susceptibility to INH (17).

In Enterobacteriaceae, *oxyR* activates specific defense mechanisms that detoxify reactive oxygen intermediates and remove their harmful products (20, 21). One of the genes controlled by *oxyR* in enteric bacteria is *ahpC*, encoding the subunit of alkyl hydroperoxide reductase that reduces organic peroxides to their corresponding alcohols (20). Significantly, *ahpC* and *oxyR* are tightly linked and divergently transcribed in *M. tuberculosis* (Fig. 1*A*) and in the majority of other mycobacteria studied (17). Point mutations in the *oxyR–ahpC* intergenic region have been noted in some strains of *M. tuberculosis*, suggesting their potential role in the emergence of INH^r variants (18, 22, 24). It has been proposed that the putative increase in AhpC in such mutants could either directly counteract INH effects (18, 22) or could act as a compensatory change enhancing the viability of *katG* mutants (18, 24). Since harmful oxygen by-products of INH metabolism have been implicated in the toxicity of INH in bacteria (5, 11, 13–16), we investigated herein whether *ahpC* plays a role in the intrinsic sensitivity of mycobacteria to INH. We demonstrate that *ahpC* expression is a critical factor contributing to the differential susceptibility of mycobacteria to INH.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. All *M. tuberculosis* and *M. bovis* strains were from the American Type

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ATCC, American Type Culture Collection; INH, isonicotinic acid hydrazide (isoniazid); INH^r and INH^s, INH-resistant and -sensitive, respectively; Km^r, kanamycin-resistant.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U57977 and U57760 for *M. tuberculosis* H37Ra ATCC25177 and its derivative ATCC35835, respectively; U57761 and U58030 for the *M. tuberculosis* H37Rv derivatives ATCC35823 and ATCC35825, respectively; U57978 and U57762 for *M. bovis* Ravenel ATCC35720 and its derivative ATCC35727, respectively; and U58031 for *M. bovis* ATCC35728).

‡To whom reprint requests should be addressed at: Department of Microbiology and Immunology, 5641 Medical Science Building II, University of Michigan Medical School, Ann Arbor, MI 48109-0620. e-mail: deretic@umich.edu.

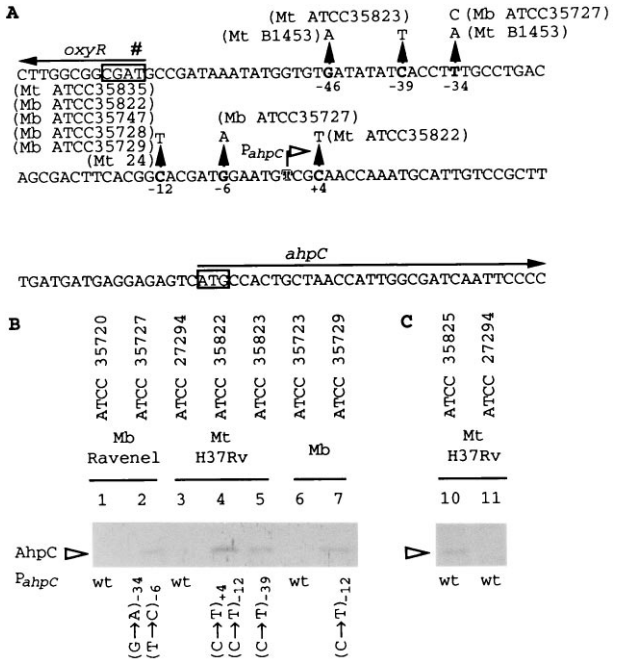

FIG. 1. (*A*) DNA sequence of the *oxyR–ahpC* intergenic region from *M. tuberculosis* and *M. bovis.* The previously reported (17, 22) and additional mutations are indicated by upward pointing triangles. Positions of the nucleotide substitutions are relative to the *ahpC* mRNA start site (Fig. 2). Strain designations are given next to the corresponding mutations. Boxed sequences, start codon of *ahpC* and the destroyed start codon (#) of *oxyR* (17). Arrows, direction of transcription. (*B*) Western blot analysis of AhpC production in three series of INH^r derivatives that carry *ahpC* promoter mutations in addition to *katG* lesions. Lanes: 2, 4, 5, 7, INH^r derivatives; 1, 3, and 6, parental INH^s strains. (*C*) AhpC production in an INH^r derivative (lane 10) of *M. tuberculosis* H37Rv (lane 11) that does not carry the *ahpC* promoter alterations. Anti-DirA antibody that recognizes mycobacterial AhpC (18, 23) was used for Western blot analysis. The strains tested are indicated above the blot and the corresponding *ahpC* promoter mutations are indicated below the blot. Mb, *M. bovis*; Mt, *M. tuberculosis*; wt, wild type.

Culture Collection (ATCC). *Mycobacterium smegmatis* mc^2 155 has been described (25). The strains VD1865-6 and VD1865-38 were two independently generated *ahpC*::Km^r mutants of *M. smegmatis* (where Km^r is kanamycin resistant). Mycobacteria were grown in Middlebrook 7H9 medium or on 7H10 plates (Difco) supplemented with 0.05% Tween 80 and ADC enrichment for *M. smegmatis* or OADC for *M. tuberculosis*. All manipulations of live *M. tuberculosis* were carried out under Biosafty Level 3 conditions. *M. tuberculosis* was inactivated by heating at 80°C for 1 h.

Recombinant DNA Techniques, Genetic Methods, and Allelic Replacements. To generate the fragment used for inactivation of *ahpC* on the *M. smegmatis* chromosome via homologous recombination, a 1.2-kb *Spe*I–*Nhe*I Km^r cassette from pMV206 was inserted into the *Bsp*EI site of the *M. smegmatis* *ahpC* gene on pDP81 (18), resulting in pDP83 with a 2.5-kb *Pst*I fragment carrying the *ahpC*::Km^r construct. This fragment was purified and introduced into *M. smegmatis* mc^2 155 by electroporation (26). Potential recombinants were selected by plating cells on 7H10 medium supplemented with 0.2% pyruvate and kanamycin at 10 μg/ml, colonies were screened for homologous recombination events by PCR, and allelic replacements were confirmed by Southern blot analysis in candidate isolates. No significant differences in catalase activities [the rate of A_{240} decrease was 0.04 ± 0.02 unit versus 0.06 ± 0.02 unit in the standard Beers–Sizer assay (27) using 25 μg of crude protein extracts] or in growth rates were observed between the parental *ahpC*$^+$ strain and its *ahpC*::Km^r derivatives.

DNA Amplification and Sequencing. The *oxyR–ahpC* intergenic region was amplified by PCR from *M. tuberculosis* and *M. bovis* using the previously described (17) oligonucleotides Ahp1P (5′-GCTTGATGTCCGAGAGCATCG-3′; positions +347 to +327 relative to the ATG of *ahpC*) and OxyR4 (5′-GGTCGCGTAGGCAGTGCCCC-3′; positions −355 to −336 relative to the *ahpC* initiation codon). The nucleotide sequences of the *oxyR–ahpC* intergenic region (positions +33 to −117, relative to the initiation codon of *ahpC*) was determined directly on the PCR products employing standard PCR DNA sequencing methods (28).

RNA Isolation and S1 Nuclease Protection Analysis. Total cellular RNA was isolated by centrifugation through a cushion of 5.7 M CsCl as described (18). Single-stranded hybridization probes for *ahpC* were prepared using plasmid pVDtb#3 (17, 18) with the *M. tuberculosis* *oxyR–ahpC* region on a 5.5-kb *Bam*HI genomic insert. The *ahpC*-specific oligonucleotide Ahp4 (5′-GGTGAAGTAGTCGCCGGGCT-3′; positions +108 to +89, relative to the *ahpC* initiation codon) was used to generate a uniformly labeled (^{32}P) single-stranded probe and to produce the corresponding sequencing ladder as described (18). Equal amounts of RNA (33 μg) were hybridized with aliquots of the radioactively labeled probe. S1 nuclease protection analysis was carried out as described (18). S1 nuclease digestion products were analyzed on sequencing gels (7.5% polyacrylamide/8 M urea/100 mM Tris/100 mM boric acid/2 mM EDTA, pH 8.3) along with the sequencing ladder. Because of the uniform labeling of single-stranded DNA probes, which dramatically improves the sensitivity of the assay, radioactive decay contributes to the presence of multiple bands corresponding to the 5′ end of mRNA, as has been noted (18).

Immunoblot Analysis. *M. smegmatis*, *M. tuberculosis*, and *M. bovis* were grown to midlogarithmic phase and crude protein extracts were obtained by homogenization in a Mini Beadbeater (Biospec Products, Bartlesville, OK) for 2 min at 2800 rpm. The cellular debris and beads were removed by centrifugation and the supernatant was mixed with an equal volume of 2× SDS/PAGE sample loading buffer and analyzed on SDS/11% polyacrylamide gels. The proteins were transferred onto Immobilon-P membranes (Millipore) by electroblotting and subjected to Western blot analysis using rabbit antiserum to DirA (AhpC) of *Corynebacterium diphtheriae* (23) that recognizes mycobacterial AhpC (18).

INH Sensitivity Determination. A previously reported (29) disk INH-inhibition assay was used with some modifications. *M. smegmatis* strains $mc^2$155 (*ahpC*$^+$) and its *ahpC*::Km^r derivatives VD1865-6 and VD1865-38, grown to equal densities, were mixed with soft 7H10 agar and plated. Paper discs (6 mm in diameter) were impregnated with 10 μl of INH (1 mg/ml) and placed on top of the solidified soft agar. Zones of growth inhibition were measured after overnight incubation at 37°C.

RESULTS

Mapping of the *ahpC* mRNA Start Site and Detection of Transcription in *M. bovis*. The occurrence of point mutations in the *oxyR–ahpC* intergenic region in *M. tuberculosis* and *M. bovis* has been reported (18, 22). These mutations have been observed in some INH^r strains, and a particularly high incidence has been noted in Δ*katG* mutant isolates (18) as summarized in Fig. 1*A*. It has been proposed that such mutations may cause increased *ahpC* transcription thus contributing to the emergence of INH^r strains by either compensating for the loss of *katG* activity (18) or directly contributing to the low-level INH resistance (18, 22). To investigate whether mutations in the putative promoter region of *ahpC* result in

detectable *ahpC* transcription, we first mapped the promoter for *M. bovis ahpC* using S1 nuclease protection analysis and RNA extracted from *M. bovis* bacillus Calmette–Guérin (BCG) Montreal ATCC35735 and its derivative ATCC35747, which carries the most common change in the *oxyR–ahpC* region ($C^{12} \rightarrow T^{12}$) (Fig. 1*A*). The expectation was that the transcript, if any, would be observed in the *ahpC* promoter mutant. The results of these experiments (Fig. 2) can be summarized as follows: (*i*) The *ahpC* mRNA 5′ end in *M. bovis* was located within the *ahpC–oxyR* intergenic region and was 42 bp upstream of the start codon of *ahpC* (Fig. 1*A* and 2). (*ii*) This finding positioned the most frequent promoter mutation (Fig. 1*A*) at position −12 relative to the mRNA start site. (*iii*) As anticipated, increased transcription was observed in the mutant derivative (Fig. 2, lane 2). (*iv*) Somewhat surprisingly, significant expression of *ahpC* was also observed (Fig. 2*A*, lane 1) in the parental strain *M. bovis* BCG ATCC35735 carrying the wild-type *ahpC* promoter.

Increased AhpC Production in *M. tuberculosis* with ahpC Promoter Mutations. We investigated *ahpC* expression at the protein level in three series of isogenic strains: (*i*) the INH^s parent *M. tuberculosis* H37Rv (ATCC27294) and its INH^r derivatives ATCC35822 [carrying a double *ahpC* promoter mutation at positions −12 and +4 (Fig. 1*A*) and a full-length *katG* deletion which is considered to be the cause of its high INH resistance (22)] and ATCC35823 [carrying an *ahpC* promoter mutation at position −39 (GenBank accession no. U57761; Fig. 1*A*) and a missense $T^{274} \rightarrow P^{274}$ mutation that renders KatG inactive (30)]; (*ii*) *M. bovis* 35723 (INH^s) and its INH^r derivative ATCC 35729 [with a typical C^{12} to T^{12} transition in the *ahpC* promoter (22) and a nonsense mutation $W^{198} \rightarrow$ Stop in *katG* (30)]; and (*iii*) *M. bovis* Ravenel ATCC35720 (INH^s) and its INH^r derivative ATCC35727 [carrying a double *ahpC* promoter mutation at positions −34 and −6 (Fig. 4*A*) and a 76-bp deletion in *katG* 106 bp downstream of the *katG* initiation codon (30)]. Western blot analyses with the antibody against DirA (AhpC) of *C. diphtheriae* (23), which recognizes mycobacterial AhpC (18), indicated that AhpC was observed only in the mutant INH^r progeny while AhpC was not detectable in any of the corresponding parental INH^s strains (Fig. 1*B*). These observations are consistent with the activation of *ahpC* expression in *ahpC* promoter mutants resulting in detectable production of AhpC.

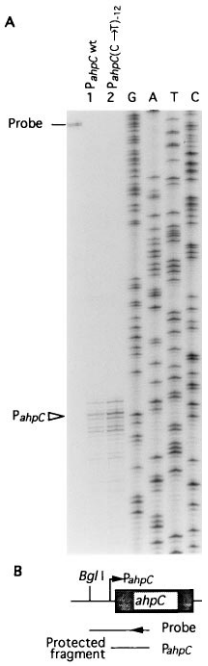

FIG. 2. S1 nuclease protection mapping of the *ahpC* mRNA 5′ end in *M. bovis*. (*A*) RNA was isolated from *M. bovis* bacillus Calmette–Guérin (BCG) ATCC35735 (lane 1) and *M. bovis* BCG ATCC35747 (lane 2). These strains have been examined for AhpC production (18) and displayed same relationships as shown in Fig. 1. Bar, location of the untreated probe. (*B*) Schematic representation of the probe and protected fragment in relationship to *ahpC* and its upstream region. Single-stranded S1 nuclease probe was generated using the same primer that served to generate DNA sequencing ladder (GATC). *Bgl*I site was the other end of the probe. Triangle (P_{ahpC}), *ahpC* mRNA 5′ end; wt, wild type; $(C \rightarrow T)_{-12}$, C → T transition at position −12 relative to the mRNA start site.

Multiple Mechanisms Lead to Enhanced AhpC Production in *M. tuberculosis*. While testing the series of strains described above, we also examined another isogenic INH^r derivative of *M. tuberculosis* H37Rv, strain ATCC35825, that has an enzymatically functional catalase peroxidase (30). This strain displays an intermediate level of INH resistance [5 μg/ml (30)] and carries the commonly observed $Arg^{463} \rightarrow Leu^{463}$ polymorphism, considered by some researchers to confer low-level resistance to INH (10, 30–33). However, this polymorphism is present in all *M. bovis* and *M. microti* strains (10) and does not affect catalase or peroxidase activity (30, 32); it has also been reported in a number of *M. tuberculosis* strains showing normal INH sensitivity (10, 34, 35). When tested for *ahpC* promoter mutations, *M. tuberculosis* ATCC35825 did not carry any changes in the *oxyR–ahpC* intergenic region. Surprisingly, we could detect AhpC by immunoblot analysis in this variant while it was absent in the parental strain H37Rv (Fig. 1*C*). These results indicate that AhpC production is increased in at least some INH^r isolates of *M. tuberculosis* that do not carry mutations in the *ahpC* promoter region. These observations suggest the existence of multiple mechanisms, besides *ahpC* promoter mutations, that can lead to enhanced production of AhpC with implications for the emergence of INH resistance in *M. tuberculosis*.

Inactivation of *ahpC* in *M. smegmatis* Causes Hypersensitivity to INH. In addition to INH-sensitive (INH^s) strains of *M. tuberculosis*, AhpC is also not detectable (18) in *Mycobacterium aurum*, a fast growing species highly susceptible to INH (18, 36). In contrast, AhpC is produced in significant amounts in *M. smegmatis*, an organism epitomizing mycobacteria with low sensitivity to INH (3, 5, 9). This suggested to us that the differences in *ahpC* expression may correlate with the intrinsic sensitivity to INH in mycobacterial species. To test this hypothesis, we insertionally inactivated the recently cloned and characterized *ahpC* gene (18) on the chromosome of *M. smegmatis* and examined INH sensitivity of the resulting strains (Fig. 3*A*). Two independent *ahpC*::Km^r mutant isolates were obtained (VD1865-6 and VD1865-38). The gene replacements were confirmed by Southern blot analysis (Fig. 3*B*). The mutant *ahpC*::Km^r strains no longer produced AhpC as determined by Western blot analysis (Fig. 3*C*). The inactivation of *ahpC* caused a striking increase in susceptibility of *M. smegmatis* to INH as illustrated in Fig. 4. While the wild-type *M. smegmatis* showed a growth inhibition zone of 25.2 ± 0.3 mm, its *ahpC*::Km^r derivatives displayed an inhibition zone of 40.3 ± 0.4 mm [*P* value (*t* test) was 9×10^{-17}]. Depending upon growth conditions, minimal inhibitory concentration of INH was decreased 4-fold to one order of magnitude for the mutant strain. The effect was specific for INH since the *ahpC*::Km^r derivatives did not show significant differences in sensitivity to other drugs such as rifampicin [23.3 ± 0.9 mm for $ahpC^+$ versus 24.0 ± 0.6 mm for *ahpC*::Km^r cells; *P* value (*t* test) was 0.6] in disk inhibition assays. These results demonstrate that AhpC contributes to the relative ability of mycobacteria to resist the action of INH.

DISCUSSION

In this work, we have demonstrated that *ahpC* plays a role in the intrinsic sensitivity of mycobacteria to INH. This was

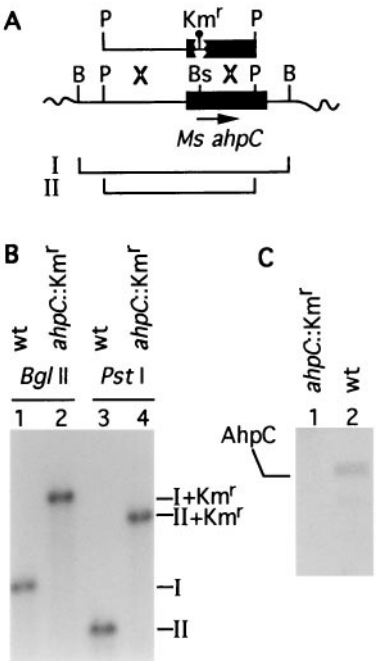

FIG. 3. Inactivation of *ahpC* on the chromosome of *M. smegmatis*. (*A*) Schematic representation of the recombinational events between chromosomal *ahpC* and *ahpC*::Km^r on a 2.5-kb *Pst*I fragment introduced into *M. smegmatis* mc^2 155 by electroporation. B, *Bgl*II; P, *Pst*I; Bs, *Bsp*EI. Balloon, 1.2-kb Km^r cassette. Lines I and II correspond to the wild-type *Bgl*II and *Pst*I fragments, respectively. (*B*) Southern blot hybridization analysis of the insertional inactivation of *ahpC* in the strain VD1865-6. Lanes: 1 and 3, *M. smegmatis* mc^2 155 *ahpC*$^+$; 2 and 4, *M. smegmatis* VD1865-6. I, 1.7-kb *Bgl*II fragment (wt); II, 1.3-kb *Pst*I fragment (wt). I+Km^r (2.9 kb) and II+Km^r (2.5 kb) are the corresponding bands hybridizing with the *ahpC* probe in the strain VD1865-6. (*C*) Western blot analysis of AhpC expression in the parent strain *M. smegmatis* mc^2 155 (wt) and its mutant derivative VD1865-6 (*ahpC*::Km^r). Equal amounts of protein from crude extracts separated by SDS/PAGE were probed with anti-DirA antibody.

accomplished by insertional inactivation of the *ahpC* gene in *M. smegmatis*, which increased the susceptibility of this organism to INH. In addition, the presented data indicate that the gene product of *ahpC* is below detection limits in all INH^s strains of *M. tuberculosis* and *M. bovis* tested but that its transcription is increased and its gene product can be detected by immunoblots in *M. tuberculosis* isolates with mutations in the *ahpC* promoter. Furthermore, mechanisms besides *ahpC* promoter mutation appear to enhance AhpC production or

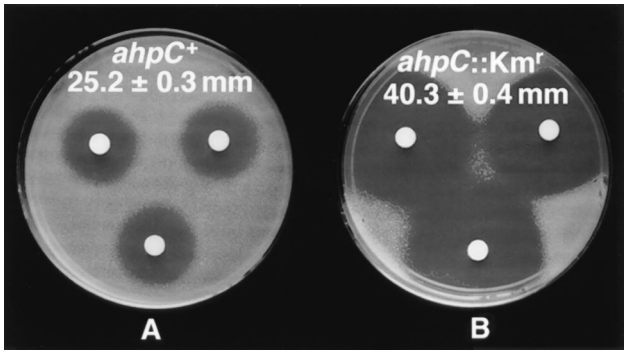

FIG. 4. Inactivation of *ahpC* causes hypersensitivity to INH in *M. smegmatis*. Shown are growth inhibition zones with INH in *M. smegmatis* mc^2 155 (*ahpC*$^+$) and *M. smegmatis* VD1865-6 (*ahpC*::Km^r). Discs were impregnated with 10 μl of INH at 1 mg/ml and placed on top of the soft agar with corresponding strains. (*A*), *M. smegmatis* mc^2 155; (*B*), *M. smegmatis* VD1865-6.

stability in some *M. tuberculosis* strains with low-level INH resistance. These findings, together with the previous demonstration of a reduced susceptibility of *M. tuberculosis* to INH upon introduction of cosmids carrying the complete *oxyR* and *ahpC* genes of *Mycobacterium leprae* (17), support the notion that the defect in oxidative stress response in the tubercle bacillus is a major contributing factor to its exceptionally high INH sensitivity.

Mycobacteria show a wide range of innate susceptibilities to INH (3, 5). At one extreme are the highly sensitive members of the *M. tuberculosis* complex, while at the other are organisms such as *M. smegmatis* that are affected only by high concentrations of INH (3, 5, 9). Within the group of INH^s organisms is *Mycobacterium aurum,* a fast growing nonpathogenic species that approaches INH susceptibility levels in *M. tuberculosis* (18, 36). In a recent analysis, AhpC could not be detected in *M. aurum*, in further support of the inverse correlation between *ahpC* expression and INH susceptibility (18). Since peroxidatic activation of INH involves production of damaging reactive oxygen intermediates (3, 11, 15, 16), it is conceivable that AhpC may counteract these effects by reducing oxidized targets or by detoxifying the activated INH. In this model, organisms with no or less AhpC may be at a disadvantage when exposed to INH.

The dysfunction of the peroxide stress response, which can be at least partially attributed to the lesions in *oxyR* (17), appears to be a major underlying cause of the exceptional sensitivity of *M. tuberculosis* to INH. Since *ahpC* is most likely only one of the genes controlled by *oxyR* [e.g., besides AhpC, eight other polypeptides inducible by peroxides have been identified in *M. smegmatis* (18)], additional putative members of the regulon may also participate in these processes. It will be of interest to determine whether such putative elements display cumulative effects in protection against INH. It is also possible that other previously appreciated peculiarities of *M. tuberculosis*, recently reviewed by Zhang and Young (5), may contribute to the overall sensitivity levels. However, such notions must await experimental support. For example, it has been speculated that the absence of the second catalase (*katE*) may be the reason for high susceptibility of *M. tuberculosis* to INH. However, it has been recently shown (37) that introduction of the second catalase (*katE*) in *M. tuberculosis* did not lower its sensitivity to INH. Although mycolic acid biosynthesis is a likely target for INH (6, 7, 12), this aspect of mycobacterial physiology alone may not suffice to explain the dramatic differences in sensitivities to INH. InhA, one of the suspected specific targets for INH (6, 7, 12), is also present in *M. smegmatis* (6) and is a close homolog of a pyridine nucleotide-linked enoyl reductase involved in the biogenesis of fatty acids in *Escherichia coli* (38), attesting to the ubiquitous presence of these factors. As an added curiosity and in keeping with our results, inactivation of *oxyR* and *ahpC* can render enteric bacteria, normally insensitive to INH, susceptible to this drug (29).

While the primary objective of our study, a relationship between *ahpC* activity and the intrinsic sensitivity to INH in several mycobacterial species, has been established, the role of *ahpC* in the emergence of INH resistance in *M. tuberculosis* is at present a controversial issue. Based on the effects that *ahpC* expression has on mycobacterial susceptibility to INH (18, 22), it appears only reasonable to expect that upregulation of *ahpC* could decrease sensitivity of *M. tuberculosis* to this drug. However, the demonstration of the role of such putative processes is complicated by the high incidence of *ahpC* promoter mutations in strains that already carry a mutation in *katG* (18). One interpretation is that AhpC compensates for the lack of *katG* expression in such isolates; alterations increasing *ahpC* expression may simply represent second-site suppressor mutation in strains that display high levels of INH resistance due to the loss of KatG (18). However, activation of

ahpC may alone confer low-level resistance to INH since complementation of *katG* in some strains with dual *katG* and *ahpC* promoter mutations does not completely restore the INH sensitivity (22). In a recent study involving a large collection of clinical isolates that will be reported separately (S. Sreevantsan, Y.S., V.D., and J. M. Musser, unpublished data), a significant number of INH^r strains have been identified carrying alterations only in the *ahpC* promoter. Out of 20 INH^r clinical isolates carrying mutations in the *ahpC* promoter region at various positions between −45 and −4 relative to the *ahpC* mRNA start site, six strains had wild-type *katG* and *inhA* alleles while an additional group of six isolates had either the common R463L polymorphism or a neutral change in *katG* along with an intact *inhA* gene. In addition, a smaller subset of INH^r strains carried missense mutation in the structural portion of the *ahpC* gene. These findings appear to support an independent role for *ahpC* mutations in the emergence of low level INH resistance in *M. tuberculosis*. However, due to the difficulty in demonstrating increased resistance to INH in *M. tuberculosis* harboring plasmid-borne *ahpC* (24), this issue remains open for further investigation.

It will also be of interest to determine the nature of processes causing increased AhpC levels in some strains that do not carry mutations in the *ahpC* promoter and whether similar mechanisms can operate in some clinical isolates with low-level INH resistance. Perhaps additional parallels concerning the regulation of oxidative stress response exist between mycobacteria and other bacteria. In Enterobacteriaceae, this system appears to include elements of posttranslational control (21) and in *C. diphtheriae* involves iron regulation (23). Further investigations of these putative regulatory mechanisms and complementary studies aimed at understanding potential consequences of the absence of *oxyR* function in *M. tuberculosis* on the course of infection and pathogenesis in tuberculosis may also shed light on host–pathogen interactions that lead to the fascinating phenomenon of the loss of *oxyR*.

We thank S. S. Tai for anti-DirA antibody and M. Schurr, L. Via, and H. Yu for their comments. This work was supported by Grant AI35217 from the National Institute of Allergy and Infectious Diseases.

1. Bloom, B. R. & Murray, C. J. L. (1994) *Science* **257,** 1055–1064.
2. Robitzek, E. H. & Selikoff, I. J. (1952) *Am. Rev. Tuberc.* **65,** 402–428.
3. Youatt, J. (1969) *Am. Rev. Respir. Dis.* **99,** 729–749.
4. Winder, F. G. (1982) in *The Biology of the Mycobacteria,* eds. Ratledge, C. & Stanford, J. S. (Academic, London), pp. 354–438.
5. Zhang, Y. & Young, D. B. (1993) *Trends Microbiol.* **1,** 109–113.
6. Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., Collins, D., De Lisle, G. & Jacobs, W. R. (1994) *Science* **263,** 227–230.
7. Dessen, A., Quemard, A., Blanchard, J. S., Jacobs, W. R. & Sacchettini, J. C. (1995) *Science* **267,** 1638–1641.
8. Middlebrook, G. (1954) *Am. Rev. Tuberc.* **69,** 471–472.
9. Zhang, Y., Heym, B., Allen, B., Young, D. & Cole, S. T. (1992) *Nature (London)* **358,** 591–593.
10. Musser, J. M. (1995) *Clin. Microbiol. Rev.* **8,** 496–514.
11. Shoeb, H. A., Bowman, B. U., Ottolenghi, A. C. & Merola, A. J. (1985) *Antimicrob. Agents Chemother.* **27,** 399–403.
12. Johnsson, K., King, D. S. & Schultz, P. G. (1995) *J. Am. Chem. Soc.* **117,** 5009–5010.
13. Kruger-Thiemer, E. (1958) *Am. Rev. Tuberc.* **77,** 364–367.
14. Winder, F. G. (1960) *Am. Rev. Respir. Dis.* **81,** 68–78.
15. Shoeb, H. A., Bowman, B. U., Ottolenghi, A. C. & Merola, A. J. (1985) *Antimicrob. Agents Chemother.* **27,** 408–412.
16. Shoeb, H. A., Bowman, B. U., Ottolenghi, A. C. & Merola, A. J. (1985) *Antimicrob. Agents Chemother.* **27,** 404–407.
17. Deretic, V., Philipp, W., Dhandayuthapani, S., Mudd, M. H., Curcic, R., Garbe, T., Heym, B., Via, L. E. & Cole, S. T. (1995) *Mol. Microbiol.* **17,** 889–900.
18. Dhandayuthapani, S., Zhang, Y., Mudd, M. H. & Deretic, V. (1996) *J. Bacteriol.* **178,** 3641–3649.
19. Sherman, D. R., Sabo, P. J., Hickey, M. J., Arain, T. M., Mahairas, G. G., Yuan, Y., Barry, C. E. & Stover, C. K. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6625–6629.
20. Christman, M. F., Morgan, R. W., Jacobson, F. S. & Ames, B. N. (1985) *Cell* **41,** 753–762.
21. Toledano, M. B., Kullik, I., Trinh, F., Baird, P. T., Schneider, T. D. & Storz, G. (1994) *Cell* **78,** 897–909.
22. Wilson, T. M. & Collins, D. M. (1996) *Mol. Microbiol.* **19,** 1025–1034.
23. Tai, S. S. & Zhu, Y. Y. (1995) *J. Bacteriol.* **177,** 3512–3517.
24. Sherman, D. R., Mdluli, K., Hickey, M. J., Arain, T. M., Morris, S. L., Barry, C. E. & Stover, C. K. (1996) *Science* **272,** 1641–1643.
25. Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T. & Jacobs, W. R. (1990) *Mol. Microbiol.* **4,** 1911–1919.
26. Jacobs, W. R., Kalpana, G. V., Cirrilo, J. D., Pascopella, L., Snapper, S. B., Udani, R. A., Jones, W., Barletta, R. G. & Bloom, B. R. (1991) *Methods Enzymol.* **204,** 537–555.
27. Beers, R. F. & Sizer, I. W. (1952) *J. Biol. Chem.* **195,** 133–140.
28. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1989) *Current Protocols in Molecular Biology* (Wiley, New York).
29. Rosner, J. L. (1993) *Antimicrob. Agents Chemother.* **37,** 2251–2253.
30. Rouse, D. A. & Morris, S. L. (1995) *Infect. Immun.* **63,** 1427–1433.
31. Cockerill, F. R., Uhl, J. R., Temesgen, Z., Zhang, Y., Stockman, L., Roberts, G. D., Williams, D. L. & Kline, B. C. (1995) *J. Infect. Dis.* **171,** 240–245.
32. Heym, B., Alzari, P. M., Honore, N. & Cole, S. T. (1995) *Mol. Microbiol.* **15,** 235–245.
33. Morris, S. L., Bai, G. H., Suffys, P., Portillo-Gomez, L., Fairchok, M. & Rouse, D. A. (1995) *J. Infect. Dis.* **171,** 954–960.
34. Rouse, D. A., Li, Z., Bai, G.-H. & Morris, S. L. (1995) *Antimicrob. Agents Chemother.* **39,** 2472–2477.
35. Pretorius, G. S., Helden, P. D., Sirgel, F., Eisenach, K. D. & Victor, T. C. (1995) *Antimicrob. Agents Chemother.* **39,** 2276–2281.
36. Lirzin, M. L., Vivien, J. N., Lepeuple, A., Thibier, R. & Pretet, C. (1971) *Rev. Tuberc. Pneumol.* **35,** 350–356.
37. Milano, A., De Rossi, E., Gusberti, L., Heym, B., Marone, P. & Riccardi, G. (1996) *Mol. Microbiol.* **19,** 113–123.
38. Bergler, H., Wallner, P., Ebeling, A., Leitinger, B., Fuchsbichler, S., Aschauer, H., Kollenz, G., Hogenauer, G. & Turnowsky, G. (1994) *J. Biol. Chem.* **269,** 5493–5496.