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Analysis of the *oxyR-ahpC* Region in Isoniazid-Resistant and -Susceptible *Mycobacterium tuberculosis* Complex Organisms Recovered from Diseased Humans and Animals in Diverse Localities

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Automated DNA sequencing was used to analyze the *oxyR-ahpC* region in 229 *Mycobacterium tuberculosis* complex isolates recently recovered from diseased humans and animals. The entire 1,221-bp region was studied in 118 isolates, and 111 other isolates were sequenced for *oxyR*, *ahpC*, or the 105-bp *oxyR-ahpC* intergenic region. The sample included isoniazid (INH)-susceptible and -resistant organisms in which the *katG* gene and *inhA* locus had previously been sequenced in their entirety to identify polymorphisms. A total of 16 polymorphic sites was identified, including 5 located in *oxyR*, 2 in *ahpC*, and 9 in the 105-bp intergenic region. All polymorphic sites located in the intergenic region, and the two missense substitutions identified in *ahpC*, occurred in INH-resistant organisms. In contrast, there was no preferential association of polymorphisms in *oxyR*, a pseudogene, with INH-resistant organisms. Surprisingly, most INH-resistant strains with KatG codon 315 substitutions that substantially reduce catalase-peroxidase activity and confer high MICs of INH lacked alterations in the *ahpC* gene or *oxyR-ahpC* intervening region. Taken together, the data are consistent with the hypothesis that some polymorphisms located in the *ahpC-oxyR* intergenic region are selected for after reduction in catalase or peroxidase activity attributable to *katG* alterations arising with INH therapy. These mutations are uncommon in recently recovered clinically significant organisms, and hence, there is no strict association with INH-resistant patient isolates. The *ahpC* compensatory mutations are apparently uncommon because strains with a KatG null phenotype are relatively rare among epidemiologically independent INH-resistant organisms.

Mycobacterium tuberculosis infects over one-third of the world's population and causes almost three million deaths every year globally (14, 20). Isoniazid (INH) is used in combination with other primary antituberculosis drugs to treat diseased patients. Several lines of evidence are consistent with the idea that mutations in the *katG* gene, encoding catalase-peroxidase, and the *inhA* locus, encoding enzymes involved in mycolic acid biosynthesis, participate in INH resistance (1, 9, 10, 15–18, 34, 35). Together, mutations occurring in these two genes may account for up to 80% of all organisms resistant to this critical antituberculosis agent (16, 18). The molecular mechanisms responsible for INH resistance in the other 20% of isolates are unknown.

Based on studies conducted with *Escherichia coli* and *Salmonella typhimurium* (3, 4, 7, 21, 22, 30), organisms naturally resistant to INH, it was speculated that mutations affecting one or more genes forming a hypothetical OxyR regulon contribute to the exquisite sensitivity of *M. tuberculosis* to this drug (22). The OxyR regulon in members of the family *Enterobacteriaceae* and other organisms is a sophisticated oxidative-stress regulatory pathway that is activated in response to environmental challenges such as treatment with hydrogen peroxide. OxyR is a regulatory protein that functions as both an oxidative-stress sensor and activator of gene transcription (7). OxyR controls expression of genes encoding detoxifying enzymes such as catalase-hydroperoxidase I and alkyl hydroperoxidase, encoded by *katG* and *ahpC*, respectively (7).

The model based on the *Enterobacteriaceae* led to the recent characterization of the apparent *oxyR* and *ahpC* homologs in *M. tuberculosis* (5, 26). Interestingly, the *oxyR* homolog in *M. tuberculosis* complex organisms was found to have numerous frameshift mutations and deletions that render it naturally inactive; that is, it is a pseudogene. This observation, along with the demonstration that some transformants of *M. tuberculosis* carrying cosmids with a fully active *oxyR-ahpC* region of *Mycobacterium leprae* were resistant to INH (5), was interpreted to be consistent with the idea that the sensitivity of *M. tuberculosis* to INH is due at least partly to an aberrant OxyR regulon.

A corollary of the hypothesis that naturally occurring INH susceptibility in *M. tuberculosis* is in part due to a defective OxyR regulon postulates that mutations in other genes found in a putative regulon could, in principle, produce INH resistance in *M. tuberculosis*. For example, it is possible that enhanced expression of alkyl hydroperoxidase encoded by *ahpC* partially or entirely compensates for a defective OxyR regulon, thereby resulting in INH resistance. Evidence consistent with this idea has been presented elsewhere (6, 32). These investigators found mutations in the *ahpC* promoter sequences in INH-resistant organisms deficient in KatG activity but not in INH-susceptible bacteria. Moreover, it was shown with *lacZ*

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reporter gene constructs (32) and Western immunoblot analysis (6) that several of the mutant alleles had enhanced transcriptional activity. It was concluded that upregulation of the *ahpC* gene may account for INH resistance in organisms lacking mutations in *katG* or the *inhA* locus. Dhandayuthapani et al. (6) have suggested that *ahpC* promoter mutations enhance gene expression and represent compensatory changes that offset detrimental effects attributable to low or reduced *katG* expression. Importantly, data presented in another recent report showed that *ahpC* promoter changes were not causally involved in INH resistance (25). These mutations were thought to represent compensatory alterations occurring as a consequence of loss of catalase-peroxidase activity. Under this hypothesis, organisms becoming INH resistant due to decreased KatG activity require a compensatory mutation resulting in upregulation of AhpC in order to survive the toxic effects of organic peroxides generated by the host macrophage.

Because the three studies cited above used laboratory-generated strains or only a limited number of clinical isolates with significant laboratory passage histories, we thought it important to address several questions by examining a large group of recent clinical isolates characterized for polymorphisms in the *katG* gene and *inhA* locus. We therefore studied the *oxyR-ahpC* region in INH-resistant and -susceptible organisms recovered from diverse localities by automated DNA sequencing. The sample included strains previously characterized by sequencing the *katG* gene and *inhA* locus in their entirety. Our analysis found that all nucleotide polymorphisms in the *ahpC* gene or *oxyR-ahpC* intervening region were represented in INH-resistant isolates. In addition, virtually all organisms with changes in *ahpC* or the intervening region were INH resistant. Moreover, increased AhpC expression was detected by immunoblot in representative organisms with defined *oxyR-ahpC* intergenic region mutations. The results are consistent with the idea that structural alterations in this region of the genome result in upregulation of AhpC in some recently isolated clinical INH-resistant strains. However, the analysis also showed that a large percentage of INH-resistant strains, including most with substitutions in amino acid 315 of KatG that significantly decrease catalase activity and confer high MICs of INH, lack *ahpC* alterations. Taken together, the data are consistent with the compensatory change hypothesis but also show that there is neither a strict correlation nor a simple relationship between KatG structural alterations and *ahpC* promoter region changes.

MATERIALS AND METHODS

Bacterial strains. The entire 1,221-bp region was studied in 118 isolates, and 111 other isolates were sequenced for *oxyR*, *ahpC*, or the 105-bp *oxyR-ahpC* intergenic region. Several parameters were used to select these 229 *M. tuberculosis* complex strains for study. First, to ascertain that our findings had widespread geographic relevance, we included organisms from intercontinental sources. Second, to permit a fuller understanding of the relationship between *katG*, *inhA* locus, and *ahpC-oxyR* region polymorphisms in INH-resistant organisms, we employed most of the strains previously characterized for sequence variation in *katG* and the *inhA* locus (18). Third, based on the idea that *ahpC* promoter region changes represent compensatory alterations that provide an advantage to KatG-deficient organisms, we selected a broad array of organisms that had *katG* codon 315 changes that confer very low catalase-peroxidase activity and high MICs of INH. Hence, we purposely overrepresented the number of strains analyzed with *katG* codon 315 mutations. Fourth, we used a convenience sample of strains in our collection classified as *Mycobacterium africanum* and *Mycobacterium microti*. Because all strains of *M. africanum* were cultured from patients in Sierra Leone, they represent a geographically biased sample. However, these organisms were recovered from unassociated patients. Fifth, the *Mycobacterium bovis* strains included in the analysis represent a random sample of a genetically and geographically diverse group of 124 organisms recently described (28) that have been recovered from a variety of animal host species and from humans. Sixth, we included in the analysis representative members of three major genotypic groups into which all isolates of *M. tuberculosis* strict sense can be assigned based on polymorphisms located in codon 463 of *katG* and *gyrA* codon 95 (29). Seventh, organisms causing pulmonary or extrapulmonary disease were included. Virtually all organisms have been subtyped by IS*6110* profiling (31), and except for the special circumstances noted below, these strains have distinct IS*6110* subtypes. Organisms in the sample had from 0 to 21 copies of IS*6110*.

The sample of 229 organisms included bacteria classified as *M. microti* ($n = 6$ isolates), *M. africanum* ($n = 9$), *M. bovis* ($n = 29$), and *M. tuberculosis* strict sense ($n = 185$). The *M. bovis* isolates were recovered from different host species and regions of the United States and Canada. The *M. microti* specimens were cultured from voles ($n = 4$) from unknown localities, a pig ($n = 1$) in The Netherlands, and a rock hyrax ($n = 1$) in South Africa. The *M. africanum* isolates were recovered from humans with tuberculosis in Sierra Leone.

The 185 *M. tuberculosis* strains were obtained from widespread global localities, including Brazil, Chile, Mexico, Venezuela, Kenya, Yemen, Philippines, Japan, The Netherlands, Romania, and the United States. This collection of *M. tuberculosis* isolates represents the range of IS*6110* subtype diversity in the species (19) and includes organisms recovered from patients with pulmonary and extrapulmonary tuberculosis. Among these 185 *M. tuberculosis* isolates studied, 70 were resistant to INH and 71 were susceptible, and the resistance phenotype for 44 isolates was unknown.

Sequencing strategy. The DNA sequence of the 1,221-bp *oxyR-ahpC* region was analyzed in 118 *M. tuberculosis* complex organisms, including *M. microti* ($n = 6$ isolates), *M. africanum* ($n = 9$), *M. bovis* ($n = 29$), and *M. tuberculosis* strict sense ($n = 74$). A three-stage sequencing strategy was used. First, a 528-bp segment containing the entire 528-bp *oxyR* gene was amplified with the following oligonucleotide primers: forward, 5′-GGTGATATATCACACCATA-3′; reverse, 5′-CTATGCGATCAGGCGTACTTG-3′. A GeneAmp System 9600 thermocycler (Perkin-Elmer Corp., Foster City, Calif.) was used with the following parameters: annealing temperature of 55°C for 21 s, extension at 72°C for 22 s, and a denaturation step at 94°C for 21 s. Each reaction was preceded by an initial denaturation step at 94°C for 60 s and terminated with a final extension step at 72°C for 5 min. DNA sequencing reactions were performed with the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.), and data were generated with an ABI 373A automated instrument.

By using the same thermocycler parameters and forward (5′-ATGAGGAGA CATATGCCAATGCTA-3′) and reverse (5′-CCCGGCCAACCGGATCCCGG TTAG-3′) primers, a 621-bp fragment containing the entire 588-bp *ahpC* gene was amplified by PCR. This region was sequenced as described for *oxyR*, with two forward primers: 5′-ATGAGGAGACATATGCCAATGCTA-3′ and 5′-CTTTT GGCCGAAAGACTTCAC-3′.

A 701-bp region containing the 105-bp *oxyR-ahpC* intergenic region was amplified by PCR with a GeneAmp System 9600 thermocycler (Perkin-Elmer Corp.). The thermocycler parameters used were denaturation at 94°C for 21 s, annealing at 60°C for 21 s, and extension at 72°C for 15 s. The 30-cycle protocol was preceded by a single denaturation step at 94°C for 60 s and ended with a final extension step at 72°C for 5 min. The primers used for PCR amplification of the intergenic region were as follows: forward, 5′-GCTTGATGTCCGAGAGCAT CG-3′; reverse, 5′-GGTCGCGTAGGCAGTGCCCC-3′.

All sequence data were assembled and edited electronically with EDITSEQ, ALIGN, and MEGALIGN programs (DNASTAR, Madison, Wis.) and compared with a published sequence (5) for the *oxyR-ahpC* region (GenBank accession number U16243).

INH susceptibility testing. Isolates of *M. tuberculosis* strict sense and *M. africanum* were tested previously for their INH resistance phenotype by the BACTEC (Becton Dickinson, Sparks, Md.) radiometric method (8, 11, 27) or the proportion method (8, 11). INH susceptibility testing of *M. microti* and *M. bovis* isolates was not conducted, but historically virtually all strains of these species are susceptible to this antimicrobial agent.

Immunoblot analysis of AhpC expression in isolates with polymorphisms in the *oxyR-ahpC* region. Immunoblot analysis was conducted as previously described (6) with polyclonal antiserum raised against AhpC (DirA) of *Corynebacterium diphtheriae.*

RESULTS

***oxyR* polymorphisms.** The *oxyR* gene was sequenced in a total of 159 organisms, including *M. tuberculosis* ($n = 115$ isolates), *M. bovis* ($n = 29$), and all 15 isolates of *M. africanum* and *M. microti*. The analysis identified five polymorphic sites in *oxyR*, and all of these were located in the proximal 63% of the 528 bp comprising the presumed pseudogene. Four of the changes were simple nucleotide substitutions and were located at positions 37, 285, 325, and 331. The fifth alteration was an insertion of four bases (GGCG) that may have been caused by a slipped-strand mispairing event (Fig. 1). In a sample of 360 *M. tuberculosis* complex strains analyzed recently, the polymor-

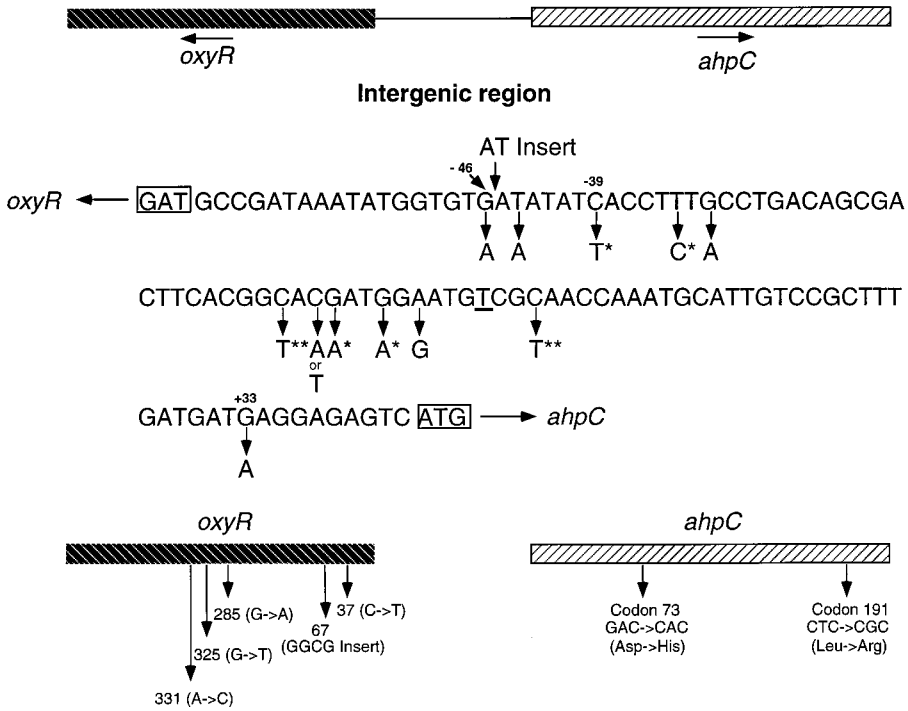

FIG. 1. Schematic of the *M. tuberculosis* complex *oxyR-ahpC* region and mutations identified. The nucleotide substitutions in the intergenic region among INH-resistant and -susceptible isolates of *M. tuberculosis* complex are depicted in the center. The bottom panel shows the nucleotide substitutions in *ahpC* and *oxyR*. Sequence polymorphisms reported by Sherman et al. (25) are designated with a single asterisk, and those sequence variants reported in both references 25 and 32 are marked with two asterisks. Numbering of intergenic region nucleotides is based on position relative to the mRNA start site (underlined nucleotide) described in reference 33.

phic site located at nucleotide 285 uniformly differentiated *M. bovis* (adenine) from non-*M. bovis* (guanine) isolates (28).

There was no apparent strict relationship between the occurrence of an *oxyR* polymorphic site and INH susceptibility or resistance. For example, the position 37 polymorphism (C→T) was found in both susceptible and resistant organisms, the nucleotide 325 change (G→T) occurred in a resistant bacterium, and the nucleotide 331 polymorphism (A→C) was identified in several susceptible isolates.

***ahpC* coding region polymorphisms.** The *ahpC* gene was sequenced in a total of 137 organisms, including *M. tuberculosis* (n = 93 isolates), *M. bovis* (n = 29), and all 15 isolates of *M. africanum* and *M. microti*. Four INH-resistant isolates of *M. tuberculosis* cultured from two patients living in El Paso, Tex., or Juarez, Mexico, had a nonsynonymous substitution in *ahpC*. Organisms recovered from the same individual were cultured 2 and 9 months after the initial isolate, whereas the fourth organism was a singleton isolate. All four organisms had the same IS*6110* subtype. The singleton strain also contained a C→T substitution at position −12 (designated relative to the mRNA start site [33]). The three isolates recovered from the same patient also had the same *katG* codon 315 (Ser→Thr) missense mutation. The singleton isolate from the second patient lacked the *katG* codon 315 change but had a synonymous (silent) substitution in codon 478 of *katG*. The mutation (GAC→CAC) found in codon 73 and would produce an Asp→His amino acid change in the AhpC protein. Sequence analysis of 13 additional strains with the same IS*6110* subtype did not identify organisms with this *ahpC* missense mutation. One additional strain had a mutation (CTC→CGC; Leu→Arg) located in codon 191.

Polymorphisms in the *oxyR-ahpC* intergenic region. The 105-bp *oxyR-ahpC* intervening region was sequenced in a total of 169 organisms, including *M. tuberculosis* (n = 125 isolates), *M. bovis* (n = 29), and all 15 isolates of *M. africanum* and *M. microti*. The intergenic region is considered a regulatory region for both *oxyR* and *ahpC* in *E. coli* (30), although in this enteric organism the two genes are not adjacent. The analysis identified nine simple polymorphic sites in the 169 organisms (Fig. 1). Simple nucleotide substitutions were identified at positions +33, −4, −6, −10, −12, −32, −39, −44, and −46 (designated relative to the mRNA start site identified in reference 33) (Table 1). In addition, one organism had a dinucleotide insertion located between positions −45 and −46. With relatively few exceptions, all strains with intervening-region mutations were INH resistant and had missense or other mutations in *katG* that would result in structural variation in KatG (Table 1).

***ahpC* promoter region changes in strains with *katG* deletions.** In three clinical isolates in our collection the *katG* structural gene was deleted, and all three had *ahpC* promoter region mutations. Two of the organisms had a C→T substitution located at position −39, and the third isolate had a T→A mutation at position −44.

Enhanced AhpC expression in some isolates with polymorphisms in *ahpC* and the intergenic region. Immunoblot detection of the *ahpC* gene product in *M. bovis* strains with *ahpC* promoter mutations has been reported previously (6). The majority of the *ahpC* promoter mutations in the previously analyzed strains are located at position −12 relative to the mRNA start site (33). Inasmuch as *M. bovis* strains with the −12 C→T mutation have detectable AhpC (Fig. 2), we examined whether AhpC could be detected in *M. tuberculosis* strict sense strains with representative mutations located at other nucleotide sites in the *oxyR-ahpC* region. Extracts made from four strains were tested (Fig. 2). One strain with a C→T sub-

TABLE 1. Nucleotide and codon polymorphisms identified in the *ahpC* and upstream *ahpC* regions of *M. tuberculosis* complex isolates

oxyR-ahpC polymorphism	*n*[a]	KatG[b] change(s)	*inhA*[c]	INH susceptibility phenotype[d]
ahpC codon (amino acid) changes[e,f]				
Codon 73 GAC→CAC (D→H)	1	315 S→T	WT	R
Codon 73 GAC→CAC (D→H)	1	WT	WT	R
Codon 191 CTC→CGC (L→R)	1	WT	WT	R
Intervening region nucleotide changes[f,g]				
+33 G→A	1	WT	WT	S
−4 A→G	1	315 S→T	WT	R
−6 G→A	1	315 S→T	WT	R
−6 G→A	4	WT	WT	R
−10 C→A	1	172 A→T	WT	R
−10 C→T	1	463L	WT	R
−10 C→T	1	393 I→N	WT	R
−12 C→T	2	463L	WT	R
−12 C→T	1	WT	WT	R
−32 G→A	1	WT	WT	R
−39 C→T	1	463L	WT	R
−39 C→T	2	Deletion	WT	R
−39 C→T	1	WT	WT	R
−44 T→A	1	Deletion	WT	R
−45 and −46 AT insertion	1	463L	WT	R
−46 G→A	4	463L	WT	S
−46 G→A	1	315 S→T; 463L	WT	R

[a] Number of isolates (all *M. tuberculosis*) with the designated change.
[b] Catalase-peroxidase (KatG) inferred amino acid changes (WT, wild type). Unless noted otherwise, all *M. tuberculosis* (strict sense) strains have an arginine in position 463.
[c] Locus encoding Mab1 and InhA (1).
[d] R, resistant; S, susceptible.
[e] Total number of isolates analyzed, 137; number INH resistant, 65; number INH susceptible, 38.
[f] The total number of isolates includes *M. bovis* ($n = 29$) and *M. microti* ($n = 6$) isolates for which the INH susceptibility was unknown.
[g] Numbering for the *ahpC* upstream nucleotides is relative to the mRNA start site for *ahpC*. Total number of isolates analyzed, 169; number INH resistant, 70; number INH susceptible, 65.

stitution located at position −39 relative to the *ahpC* mRNA start site (33) had a significant amount of AhpC detectable by the immunoblot strategy used. In contrast, extracts prepared from the other three strains tested lacked detectable AhpC by the immunoblot assay. One strain assayed had a G→A substitution located at position +33, close to the putative *ahpC* ribosomal binding site, but this mutation apparently did not sufficiently enhance AhpC production under the conditions tested. Interestingly, the INH-susceptible organism with a G→A substitution located at −46 also lacked detectable AhpC production. An INH-resistant strain (B1453) studied previously (6) expressed detectable AhpC, but this organism also contains an additional variant nucleotide (T→A) located at position −34. Taken together, the results show that in addition to the intergenic region mutations already described, other nucleotide substitutions located in this region can enhance AhpC expression. However, clearly not all strains with intergenic region substitutions have a detectable increase in AhpC levels under the conditions assayed.

DISCUSSION

The results of our analysis of this large sample of contemporary clinical isolates from widespread geographic regions extend knowledge about the occurrence of sequence variation in the *oxyR-ahpC* region in INH-susceptible and -resistant *M. tuberculosis* strains. Importantly, the study confirms limited data showing that *ahpC* promoter region mutations occur in organisms deficient in KatG activity that are INH resistant (6, 25). However, these changes are clearly rare and not obligatory in INH-resistant organisms. They apparently do not occur at a high frequency among INH-resistant clinical isolates, because strains with a KatG null phenotype are uncommon (18). The relative lack of *oxyR-ahpC* intervening region mutations in our clinical isolates compared with the laboratory-generated or -derived organisms is reminiscent of an earlier observation (12, 18) that the INH resistance-conferring mutation identified in the *inhA* gene in the laboratory has yet to be identified among large samples of clinical isolates studied, although other InhA substitutions have (12, 16, 18). Taking these results together, it appears to be critical in drug resistance studies to examine a broad spectrum of epidemiologically unassociated recent patient isolates from diverse geographic regions in order to obtain full insight into the relative importance of particular allelic variants in natural populations.

Inasmuch as our study found that *ahpC* promoter region mutations were rare in INH-resistant organisms and the Sherman et al. (25) study reported their occurrence in all eight strains examined, it is important to identify possible reasons for these frequency differences. We believe that one critical difference was the nature of the samples studied. First, our analysis examined recent clinical isolates whereas Sherman et al.

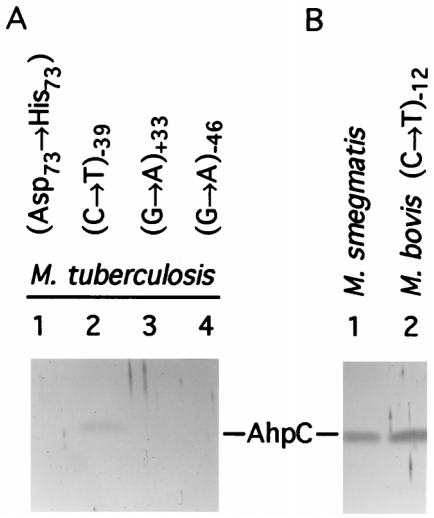

FIG. 2. Western blot analysis of *ahpC* expression in representative *M. tuberculosis* strains with variant nucleotides in the *oxyR-ahpC* region. (A) Proteins in crude cell extracts (4.8 μg of protein per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western blot analysis with antibody directed against AhpC (DirA) of *C. diphtheriae* which also recognizes mycobacterial AhpC (6). Lanes contain four different representative *M. tuberculosis* clinical isolates with alterations in the AhpC structural gene or regulatory region. The numbering system for the intergenic region changes is based on position relative to the mRNA start site described in reference 33. (B) Western blot with control samples (3.8 μg of crude protein per lane) of *M. smegmatis* mc^2155 (lane 1) and a derivative of *M. bovis* ATCC 35747 (lane 2) previously shown to produce detectable levels of AhpC (6). The *M. bovis* strain has a C→T substitution located at position −12 relative to the mRNA start (33). This mutation is not present in the parental organism, which has AhpC levels below the detection limit by immunoblot analysis (6).

(25) studied largely organisms that were derivatives of common laboratory strains such as H37Ra, H37Rv, and *M. bovis* BCG (24). These strains have been passaged extensively. We agree with the concern noted elsewhere (25) that some *ahpC* promoter region mutations may confer a premium to the organism during extensive in vitro cultivation. Under this hypothesis, mutations that result in increased expression of AhpC may, for example, enhance the growth rate or survival of the derivative relative to the progenitor. Second, we studied organisms with a broad array of distinct mutant *katG* alleles that have a range of residual KatG activity (9, 23). It is possible that the likelihood of selecting *ahpC* promoter region mutations varies depending on the residual KatG activity or other strain parameters. A related possibility is that the earlier group of organisms did not represent a random sample of INH-resistant strains causing disease in humans. Many of the strains used in the earlier study were preselected based on being catalase deficient (24). The difference in the data sets may be that the critical parameter in selection of compensatory *ahpC* mutations is decreased or absent peroxidase activity rather than simply decreased catalase activity. Under this hypothesis, certain mutations in *katG* may preferentially alter peroxidase activity or catalase activity; data consistent with this hypothesis have recently been published (23). Depending on the residual enzyme activity, varying degrees of selective pressure favoring the emergence of mutations resulting in enhanced AhpC activity or other second-site compensatory mutations could be exerted. Additional experiments are under way to test these ideas.

Relative paucity of *ahpC* promoter mutations in *katG* codon 315 mutants. It is of interest that *ahpC* promoter mutations were not present in a substantial percentage of strains containing missense changes in *katG* codon 315. Strains with substitutions of the serine residue located at position 315 of KatG have been reported to have a 20-fold decrease in catalase-peroxidase activity compared to wild-type organisms and are associated with high MICs of INH (9). A more recent study using *M. bovis* transformed with defined mutant *katG* variants confirmed that the S315T substitution reduced both catalase and peroxidase activity and conferred an INH MIC of 90 μg/ml (23). If *ahpC* promoter mutations are selected in vivo to compensate for KatG deficiency, it seems logical that an inverse relationship ought to exist between the likelihood of *ahpC* promoter mutations and catalase-peroxidase deficiency. We therefore anticipated finding that most or virtually all strains with *katG* codon 315 missense mutations would have *ahpC* promoter mutations. However, our analysis showed that this was not the case and hence demonstrates that there is clearly no simple relationship between these two parameters. We note that bacteria with all known codon 315 variants were represented in our analysis, including Ser315Thr, Ser315Asn, and Ser315Ile substitutions. As alluded to above, one potential explanation for the discrepant results is that the amino acid replacements that we found at position 315 preferentially alter catalase activity relative to the peroxidase activity of KatG. In this regard, it is noteworthy that Rouse et al. (23) have recently presented data consistent with this hypothesis. In their studies conducted with *M. bovis* BCG transformed with defined mutant *katG* alleles, the S315T variant had 60% peroxidase activity relative to the wild type but only 40% catalase activity relative to the wild type. Until additional data are available, we believe that the most likely explanation for the lack of *ahpC* promoter mutations is that position 315 changes provide adequate residual enzyme activity and hence do not require compensatory AhpC upregulation for survival.

***ahpC* promoter mutations and relative virulence.** An important question is whether *ahpC* promoter mutations affect the relative virulence of the organism. Although our study did not directly address this issue, among the strains characterized was a random sample of six organisms identified by IS*6110* restriction fragment length polymorphism analysis and characterization of antibiotic resistance-conferring mutations as being members of the so-called W (or W family of) multidrug-resistant strains (2). The organisms are invariably resistant to rifampin, INH, ethambutol, streptomycin, and sometimes ethionamide, kanamycin, capreomycin, or ciprofloxacin. These organisms form a genetically allied group of *M. tuberculosis* strains that have shared a recent clonal origin and have caused approximately 300 cases of tuberculosis in patients in New York City. In addition, W and W-family bacteria have recently spread to Pennsylvania, Ohio, South Carolina, Georgia, Florida, Colorado, and Paris, France (2, 19). These bacteria form the largest documented cluster of epidemiologically related multidrug-resistant *M. tuberculosis* cases (2). Since these organisms have a *katG* codon 315 mutation (Ser→Thr) and decreased catalase-peroxidase levels and clearly can cause abundant disease episodes, we expected to frequently identify *ahpC* promoter mutations conferring compensatory levels of AhpC activity. However, none were found, and moreover, sequence analysis of the entire *ahpC* coding region failed to identify mutations. Hence, we conclude that *ahpC* promoter region mutations are not requisite participants in the ability of INH-resistant organisms to efficiently spread from person to person rapidly and cause disease. Stated another way, INH- or multidrug-resistant strains lacking *ahpC* mutations remain important public health threats.

Many upstream mutations apparently alter *ahpC* promoter strength. It is noteworthy that virtually all of the mutations in the *oxyR-ahpC* intervening region identified were G↔C to A↔T transitions, substitutions that are expected to enhance promoter activity. Although we did not directly assay changes in promoter strength by gene fusion analyses, the results of the AhpC Western blot analysis are consistent with increased transcription of the *ahpC* gene in some strains. The data confirm and extend the recent results (25, 32) showing increased promoter activity with luciferase and β-galactosidase reporter gene fusion constructs, respectively. We note that although these investigators reported an almost 20-fold increase in promoter activity caused by substitution of two noncontiguous nucleotides in the *ahpC* promoter region of strain ATCC 35822 (renumbered as MTB35822 in reference 25), this variant was not identified in the large sample of clinical isolates examined in our study.

Virtual absence of *ahpC* coding region missense mutations. Sequence variation in the *ahpC* coding region was extremely rare in our sample and was found in only five INH-resistant isolates cultured from three patients. Four of the organisms had the same IS*6110* subtype and were recovered from patients in the sister cities of El Paso, Tex., and Ciudad Juarez, Mexico. The occurrence of the same IS*6110* subtype and the same otherwise rare *ahpC* coding change strongly suggests that these organisms have a common ancestor. Because the strains share the same rare *ahpC* coding region substitution but have different *katG* changes and *ahpC* upstream sequences, it is most likely that the *ahpC* codon 73 mutation is an ancestral condition in these four isolates. That is, the *ahpC* codon 73 mutation preceded the origin of the *katG* and *ahpC* upstream changes. The absence of the codon 73 mutation in 13 additional epidemiologically unrelated strains with the same IS*6110* subtype supports this idea. The coding region mutation (GAC→CAC) would produce an Asp→His change at amino acid 73 and is

expected to alter the pI of the resulting AhpC protein. Similarly, the Leu→Arg substitution at position 191 may also alter the pI of AhpC. It is therefore possible that these substitutions have functional significance. Currently, not enough information is available about structure-function relationships in *M. tuberculosis* AhpC or homologs in other bacteria to make an informed prediction about the functional effect of these substitutions. Experiments are under way to study this issue in more detail.

We have noted previously (13) that *M. tuberculosis* strains recovered from global sources have a rarity of synonymous (silent) nucleotide substitutions in structural genes. This observation led to the advancement of the hypothesis that the species is evolutionarily new, perhaps having originated and spread widely as recently as 15,000 to 20,000 years ago (13). The data presented here for *ahpC* extend data demonstrating that natural isolates of *M. tuberculosis* from diverse geographic localities have greatly restricted levels of synonymous substitutions in structural genes and thereby strengthen the postulate of relatively recent origin and spread.

Role of *ahpC* promoter mutations in INH resistance. Based in part on the ability of the *ahpC* gene to confer low-level INH resistance on *Mycobacterium smegmatis* (32), it has been suggested (6, 32) that increased expression of AhpC participated in conferring INH resistance to some *M. tuberculosis* strains. This interpretation was not favored by Sherman et al. (25), who concluded that AhpC upregulation does not play a direct role in INH resistance. It may well require substantial additional work to resolve this controversy, because the outcome may vary depending on the particular mutant allele used to construct the isogenic pairs and the parental strain involved. Nevertheless, taken together, our data clearly demonstrate that most strains with *ahpC* promoter region mutations have lesions at other loci (*katG* or *inhA*) that may readily explain the INH resistance phenotype. This observation argues against a direct role in INH resistance for most *ahpC* promoter changes and thereby leaves open the issue of the molecular mechanism responsible for INH resistance in organisms lacking *katG* and *inhA* locus mutations. The full spectrum of biological relevance, if any, of *ahpC* mutations and sequence variation in the *oxyR-ahpC* intergenic region is an area that needs careful investigation.

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