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Effects of Peroxides on Susceptibilities of *Escherichia coli* and *Mycobacterium smegmatis* to Isoniazid

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***Escherichia coli* strains were previously found to be susceptible to the antituberculosis drug isonicotinic acid hydrazide (isoniazid [INH]) when they carried certain mutations that also sensitize them to peroxides: a deletion in *oxyR*, a redox-sensitive regulator of hydrogen peroxide-inducible genes, or mutations in both *katG* and *ahpCF*, OxyR-regulated genes encoding hydroperoxidase I, and an alkyl hydroperoxide reductase. To test whether INH, like peroxides, activates OxyR, the effect of INH on OxyR regulation was examined. Primer extension assays showed that transcription of the OxyR-regulated *oxyS* gene was not significantly induced by INH in wild-type cells, indicating that INH does not activate OxyR. However, the INH-susceptible *katG ahpCF* mutant strain was found to have constitutively high levels of *oxyS* transcription. This suggested that the lack of peroxidase expression in these strains allows endogenous oxidants to accumulate, and this leads not only to constitutive OxyR activation but also to INH susceptibility. Consistent with this concept, hydrogen peroxide or cumene hydroperoxide potentiated the INH susceptibilities of wild-type cells, while the antioxidant ascorbic acid protected the susceptible *katG ahpCF* mutant strain from INH. Superoxide radicals, generated by paraquat, did not enhance the INH susceptibilities of wild-type cells. Hydrogen peroxide also potentiated the INH susceptibilities of susceptible and resistant (*katG* mutant) *Mycobacterium smegmatis* strains. Our results suggest that INH is converted to a more active drug by reaction with peroxides and that the INH susceptibilities of enterobacteria and mycobacteria are mechanistically related.**

The increased incidence of tuberculosis in the United States and Europe, the emergence of multiply drug-resistant *Mycobacterium tuberculosis*, and the susceptibilities of human immunodeficiency virus-infected individuals to *M. tuberculosis* have elicited new interest in antituberculosis drugs. Isonicotinic acid hydrazide (isoniazid [INH]) has been a potent and clinically important antituberculosis drug since its introduction in 1952 (2), but the emergence of INH-resistant mutants has limited its efficacy (10). While neither the mode of action nor the cellular target of INH has been elucidated, it has been suggested that peroxides and peroxidases are needed to activate INH (14, 17) and that the in vivo target may be mycolic acid synthesis (5, 15).

A new perspective on INH action has come from the recent identification of INH-susceptible mutants of *Escherichia coli* and *Salmonella typhimurium* (6). While these enterobacteria are normally highly resistant to INH, OxyR regulon mutants which are defective in the adaptive response to peroxides (see reference 13 and references therein) are susceptible to INH. Deletion strains lacking OxyR, the transcriptional activator of the regulon of hydrogen peroxide-inducible genes, were the most susceptible. Strains lacking *ahpCF*, the OxyR-regulated genes encoding an alkyl hydroperoxide reductase, showed intermediate susceptibilities, and a strain with both the *ahpCF* deletion and a Tn*10* insertion in *katG*, which encodes the OxyR-regulated hydroperoxidase I, resembled the *oxyR* deletion mutants in their INH susceptibilities. In contrast to *M. tuberculosis*, which *katG* mutations render INH resistant (17), *E. coli* mutants defective in *katG* alone did not show altered susceptibilities to INH.

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The observation that OxyR-regulated antioxidant activities provide INH resistance suggests two possible mechanisms of INH action. First, INH might act like a peroxide and activate OxyR, thereby leading to increased levels of expression of the protective hydroperoxidase I and alkyl hydroperoxide reductase activities. In either the *oxyR* or the *katG ahpCF* mutant strains there would be insufficient protective peroxidase expression and the cells would be INH susceptible. Second, INH might require activation by peroxides to generate a toxic compound (7). In this case, the role of the OxyR-peroxidase system would be to restrict the levels of peroxides to less than those necessary for INH activation. In the INH-susceptible mutants, the low levels of the peroxidases would allow peroxides to accumulate to concentrations sufficient to promote INH action. The effects of INH on OxyR reported here are consistent with the second model.

MATERIALS AND METHODS

Strains. The *E. coli* strains used were wild-type K-12, TA4315 (*ahp*Δ*5*), N7900 (*katG17*::Tn*10*), N7901 (*katG17*::Tn*10 ahp*Δ*5*), and TA4110 (*oxyR2*) (3, 6). The *Mycobacterium smegmatis* strains were MC^2155 (wild type) (9) and BH1, a *katG* INH-resistant mutant (4). Cells were cultured and plated on LB medium at 37°C as described previously (6). *M. smegmatis* cultures were briefly treated with 1% Tween 80 prior to dilution and plating.

RNA isolation and primer extension assays. Cells were grown in LB medium to an A_{600} of 0.3 to 0.4 and were then treated as indicated in Results. Total RNA was isolated by using hot phenol, and 0.1 pmol of an end-labeled oligonucleotide was annealed to 3 μg of total RNA, as in the short protocol described previously (11). The extension reaction was performed with Superscript reverse transcriptase from Gibco BRL in the reaction buffer provided by the manufacturer, and the products were separated on an 8% sequencing gel.

Susceptibility to INH. The effects of hydrogen peroxide, cumene hydroperoxide, and paraquat on INH susceptibility were tested by adding appropriate amounts of the compounds to the cells in the 2.5 ml of top agar used for plating. The concentration of compound required to reduce the efficiency of plating (EOP) by 50% is reported as the 50% inhibitory concentration (IC_{50}). INH, ascorbic acid, cumene hydroperoxide, and paraquat were obtained from Sigma Chemical Co. (St. Louis, Mo.), and hydrogen peroxide was from Fisher Scientific (Fair Lawn, N.J.).

RESULTS

Lack of OxyR activation by INH. Upon oxidation, the OxyR regulator activates the expression of defense genes which include *katG*, *ahpCF* (13), and a gene encoding a small nontranslated RNA designated *oxyS* (1). To determine whether INH acts to oxidize OxyR, leading to the induction of OxyR-regulated genes, the expression of the *oxyS* gene in response to INH was assayed. Wild-type K-12 cells were grown to the logarithmic phase in LB medium and were then treated with 200 μM hydrogen peroxide or 10, 100, and 1,000 μg of INH per ml. Total RNA was isolated at intervals after the treatments and was assayed by primer extension by using an oligonucleotide complementary to the *oxyS* RNA. The cells treated with 200 μM hydrogen peroxide showed a dramatic increase in the level of *oxyS* expression within 10 min of treatment with hydrogen peroxide. In contrast, no induction was observed when the wild-type K-12 strain was exposed to 10 μg (data not shown) or 100 μg of INH per ml, and only a faint signal was observed after 10 min of treatment with 1,000 μg of INH per ml (Fig. 1A), indicating that INH does not significantly activate OxyR.

OxyR activation in peroxidase-deficient strains. In a parallel experiment, the basal level of *oxyS* expression was examined in untreated wild-type K-12 and in *katG* (N7900), *ahpCF* (TA4315), and *katG ahpCF* (N7901) mutant strains (Fig. 1B). Previous experiments showed that the *ahpCF* mutant strain was somewhat susceptible to INH, while the *katG ahpCF* mutant strain was even more susceptible to INH (6). Total RNA was isolated from the four strains grown to the mid-logarithmic phase in LB medium and was then assayed by primer extension. The wild-type strain (K-12) and the *katG* mutant strain (N7900) showed no detectable *oxyS* expression, while the *ahpCF* mutant strain (TA4315) showed elevated levels of *oxyS* (more than 25-fold higher). The *katG ahpCF* mutant strain (N7901) showed even higher levels of *oxyS* transcription (greater than 100-fold compared with that of the wild type). This level of expression was not increased by adding 100 μg of INH per ml for 10 min (data not shown). Thus, the levels of *oxyS* transcription paralleled those of INH susceptibility seen before. These results suggested that the *ahpCF* and the *katG ahpCF* mutant strains are in a constant state of oxidative stress and that OxyR is constitutively activated because the peroxides produced by normal aerobic metabolism are not dissipated. The greater induction of *oxyS* observed in the *ahpCF* mutant strain compared with that observed in the *katG* mutant strain was surprising but may have been due to the dissipation of metabolically produced hydrogen peroxide by the alkyl hydroperoxide reductase in the *katG* mutant strain. Constitutive activation of OxyR is not required for INH susceptibility since the *oxyR*-deleted strain GSO-8 is INH susceptible (6). Thus, it is more likely that the susceptibilities of these strains to INH results from the accumulation of abnormally high levels of peroxides.

Potentiation of INH susceptibility by peroxides. If high peroxide concentrations are necessary for INH susceptibility, it might be possible to sensitize wild-type cells by treating them with exogenous peroxides. This possibility was tested by measuring the EOP of wild-type cells on LB agar with various concentrations of hydrogen peroxide in the presence or absence of normally nonlethal concentrations of INH (Fig. 2A). In the absence of peroxide, 200 μg of INH per ml did not significantly inhibit colony formation. In the absence of INH, the IC_{50} of hydrogen peroxide was about 255 μM. However, in the presence of 200 μg of INH per ml, only 80 μM hydrogen peroxide was needed for 50% inhibition of EOP. Thus, INH and hydrogen peroxide act synergistically to decrease the level of survival of wild-type bacteria.

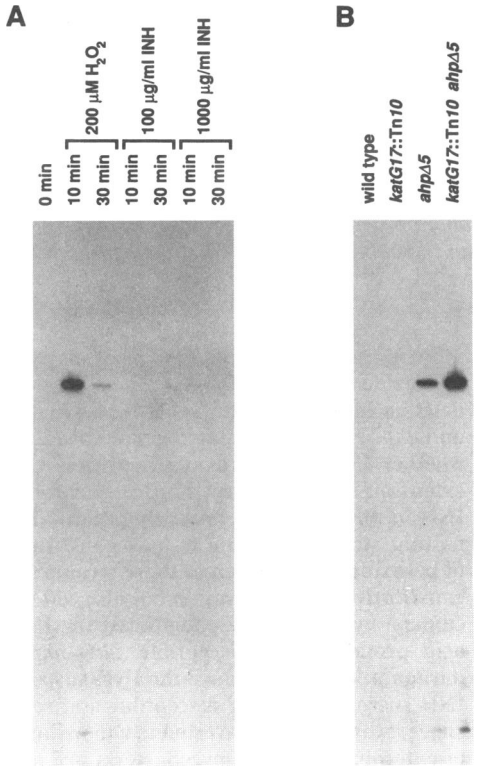

FIG. 1. Primer extension assays of *oxyS* expression. (A) Effect of INH. Total RNA was extracted from logarithmic-phase wild-type cells before and following 10 and 30 min of treatment with the indicated concentrations of hydrogen peroxide or INH. (B) *oxyS* expression in the untreated wild type and in *katG17*::Tn*10*, *ahp*Δ*5*, and *katG17*::Tn*10* *ahp*Δ*5* mutants. Total RNA was extracted from strains K-12, N7900, TA4315, and N7901 and was used for primer extension analysis as described in Materials and Methods.

Since the *ahpCF* locus plays an important role in INH susceptibility and in the detoxification of alkyl hydroperoxides (6, 12), the effect of cumene hydroperoxide on INH susceptibility in wild-type bacteria was also tested (Fig. 2B). As was found with hydrogen peroxide, cumene hydroperoxide and INH acted synergistically. The IC_{50} of cumene hydroperoxide in the absence of INH was about 300 μM (data not shown). When both 200 μg of INH per ml and 75 μM cumene hydroperoxide were present, the EOP was reduced by 50%, whereas either dose alone did not inhibit colony formation. These results show that both hydrogen and alkyl hydroperoxides can sensitize wild-type cells to INH.

If peroxides can enhance INH susceptibility in wild-type cells, a higher concentration of peroxides should be required in

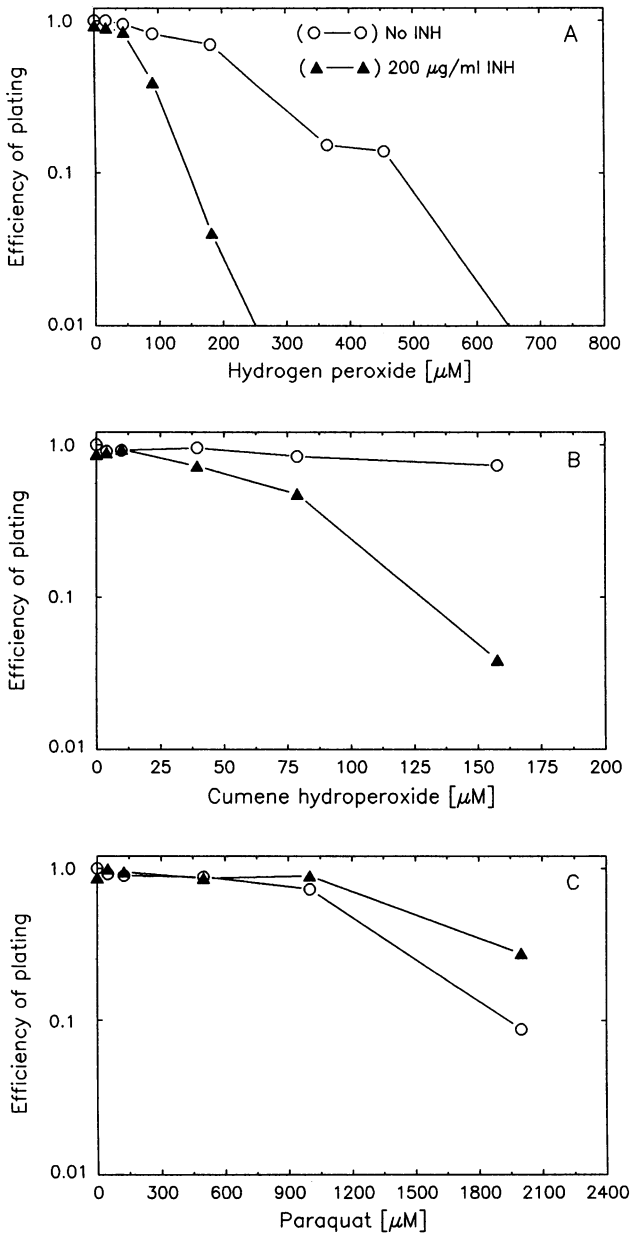

FIG. 2. Effects of hydrogen peroxide (A), cumene hydroperoxide (B), and paraquat (C) on the susceptibility of wild-type *E. coli* to 0 or 200 μg of INH per ml.

an *oxyR* constitutive mutant which expresses *katG* and *ahp* at high levels. The IC_{50} of hydrogen peroxide was about 2,400 μM in the peroxide-resistant *oxyR2* constitutive strain TA4110. As was found previously (6), TA4110 was fully resistant to 200 μg of INH per ml. The combination of only 225 μM hydrogen peroxide and 200 μg of INH per ml reduced the EOP by 50%. Thus, almost three times more hydrogen peroxide was required for synergy with INH in the *oxyR2* constitutive strain than in the wild-type strain (225 μM compared with 80 μM). Similarly, INH and cumene hydroperoxide synergistically inhibited colony formation by the *oxyR2* constitutive strain, although, again, higher concentrations of cumene hydroperoxide were required (120 μM compared with 70 μM for the wild-type strain).

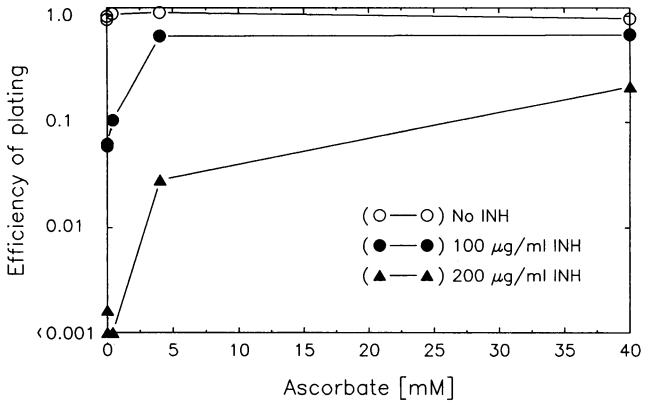

FIG. 3. Effect of ascorbate on the susceptibility of N7901 (*ahpCF katG* mutant) to 0, 100, or 200 μg of INH per ml.

Superoxides (O_2^-) are another species of reactive oxygen that are formed in the cell as a result of oxidative metabolism. The previous observation that a superoxide-susceptible strain with a *soxRS* deletion was not more INH susceptible than the wild type suggested that superoxides are not involved in INH susceptibility (6). This was further substantiated by the observation that wild-type or *oxyR2* constitutive mutant cells treated with the superoxide-generating agent paraquat did not show greater susceptibility to INH (Fig. 2C and data not shown). However, since peroxides are slowly generated from superoxides by the action of superoxide dismutases, it seemed possible that superoxide-generating agents could enhance INH susceptibility in the INH-susceptible strain N7901. When this *katG ahpCF* mutant strain was incubated with sublethal concentrations of paraquat and INH, synergy between the compounds was observed (data not shown). This supports the conclusion that peroxides but not superoxides are needed for synergism with INH.

Protection against INH susceptibility by ascorbate. If elevated peroxidative stress increases INH susceptibility, lowering of the level of this stress should decrease INH susceptibility. Therefore, the INH susceptibility of the *katG ahpCF* mutant strain N7901 was measured in the presence of ascorbic acid, a strong antioxidant (Fig. 3). In the absence of ascorbate, 100 μg of INH per ml reduced the EOP to less than 0.07. The presence of 12 mM ascorbate, however, increased the EOP to approximately 0.40 times that of the untreated controls. Even in the presence of 200 μg of INH per ml, 40 mM ascorbate increased the EOP from <0.001 to more than 0.20. Thus, ascorbate substantially reversed the lethality of INH. A similar protective effect was seen when high concentrations of glutathione were added (data not shown).

Potentiation of INH susceptibility by hydrogen peroxide in *M. smegmatis*. The results presented here strongly suggest that the *E. coli katG*-encoded peroxidase and *ahpCF*-encoded reductase prevent INH susceptibility by limiting cellular peroxide levels. The saprophytic mycobacterium *M. smegmatis* is INH susceptible, but in contrast to *E. coli*, *M. smegmatis* mutants that have lost their *katG* function are INH resistant (4). To determine whether the INH susceptibility of *M. smegmatis* is also potentiated by peroxides, the wild type and a *katG* mutant were tested in the presence of various concentrations of hydrogen peroxide. In both wild-type and *katG* mutant *M. smegmatis* strains, INH susceptibility was enhanced by hydrogen peroxide (Fig. 4). Thus, as was found with *E. coli*, peroxides are a limiting factor in the INH susceptibility of *M.*

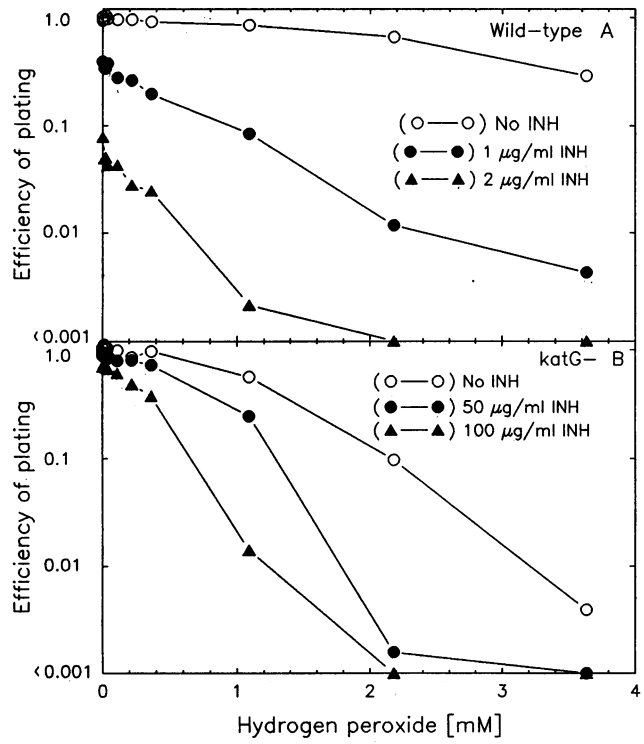

FIG. 4. Effect of hydrogen peroxide on the INH susceptibility of *M. smegmatis*. (A) Wild type (MC^2155) treated with 0, 1, or 2 μg of INH per ml. (B) *katG* mutant (BH1) treated with 0, 50, or 100 μg of INH per ml. Plates were incubated at 37°C for 3 to 4 days before colonies were counted. The *katG* mutant was about 100-fold more INH resistant but was only about 3-fold more peroxide susceptible (because of the presence of a second catalase) than the wild type (data not shown).

smegmatis. This suggests that the INH susceptibilities of enterobacteria and mycobacteria are mechanistically related.

DISCUSSION

A link between peroxide and catalase-peroxidase levels and INH toxicity has long been suspected. The present experiments strongly support the hypothesis that peroxides increase INH susceptibility and suggest that peroxides are involved in the conversion of INH into a more toxic species in both *E. coli* and *M. smegmatis*. Alternatively, the peroxides might sensitize some cellular target to the action of INH. An important direction for future studies will be to identify this activated INH species or target, which may help in the design of more potent INH derivatives. It may also be possible to increase the efficacy of INH by increasing the peroxide stress on the target bacteria.

While it is conceivable that INH could be oxidized directly by peroxides, it is likely that enzymes are involved (7). *katG*-encoded peroxidases have been found to be critical for the INH susceptibilities of *M. smegmatis* and *M. tuberculosis* (16, 17). Nevertheless, other peroxidases may contribute to INH susceptibility since even in the *katG* mutant strain *M. smegmatis* BH1 the residual INH susceptibility was enhanced by hydrogen peroxide (Fig. 4). The activity that carries out this putative peroxidation of INH is not known for *E. coli*, but its synthesis does not require OxyR since *oxyR*-deficient mutants are INH susceptible (6). In contrast to *M. smegmatis* and *M. tuberculosis*, the INH susceptibilities of *E. coli* and *S. typhimurium* are diminished, not enhanced, by their *katG*-encoded (and *ahpCF*-encoded) activities. This paradox could be explained if the different *katG*-encoded proteins differed in relative catalase-to-peroxidase activity or in specificity for INH. Shoeb et al. (8) suggested that superoxide is generated during the in vitro oxidation of INH and might mediate the toxic effects of INH. However, the present results confirm the previous suggestion (6) that superoxide is not an important component of the INH-mediated toxicity. The efficacy of INH was not increased either in strains lacking activators of superoxide dismutase expression (*soxRS*) or by the presence of increased concentrations of superoxide generated by treatment with paraquat.

The target of INH toxicity in *E. coli* is not known. A new INH (and ethionamide) resistance gene, *inhA*, has been reported recently for *M. smegmatis* and other mycobacteria (1a). This gene is thought to be involved in mycolic acid synthesis, since overexpression of *inhA* confers partial resistance to the inhibition of mycolic acid synthesis by INH in vitro (1a). Since *E. coli* and *S. typhimurium* can be INH susceptible yet do not synthesize mycolic acids, INH must affect some other target in these enterobacteria. No role in the INH susceptibility of *E. coli* has yet been found for *envM*, an *E. coli* gene with homology to *inhA* (6b). INH has been suspected of interfering with pyridoxine or nicotinic acid metabolism since pyridoxine deficiency is seen in patients treated with INH and because INH is an analog of nicotinic acid. This suggested that INH may also subject bacteria to pyridoxine deficiency. However, no sparing effect of pyridoxine or nicotinic acid was observed when the *katG ahpCF* mutant strain (N7901) was plated on glucose-minimal medium with 200 μg of INH per ml (6a). Thus, it is not likely that INH susceptibility in *E. coli* is due to interference with pyridoxine or nicotinic acid metabolism. The identification of targets for activated INH in the susceptible *E. coli* strains is now being undertaken.

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