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# Susceptibilities of *oxyR* Regulon Mutants of *Escherichia coli* and *Salmonella typhimurium* to Isoniazid

JUDAH L. ROSNER*

*Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892*

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***Escherichia coli* and *Salmonella typhimurium* are normally resistant to >500 μg of the antituberculosis drug isonicotinic acid hydrazide (isoniazid; INH) per ml. Susceptibility to INH (<50 μg/ml) has now been found for mutants that are deficient in OxyR, the oxidative stress response regulator. Two OxyR-regulated enzymes, alkyl hydroperoxide reductase and hydroperoxidase I, were identified as playing important roles in INH resistance. OxyR regulon mutants should be useful for identifying other determinants of INH resistance in both *E. coli* and *Mycobacterium tuberculosis* and for finding new INH-like drugs.**

While the incidence of tuberculosis is increasing dramatically, the efficacy of the most clinically useful drug, isonicotinic acid hydrazide (isoniazid; INH), is compromised by the emergence of resistant strains of *Mycobacterium tuberculosis* (16). Moreover, uncertainty as to the mode of action of INH hampers development of more potent derivatives. The low growth rate of *M. tuberculosis*, the risks it poses for laboratory workers, and insufficient genetic and biochemical knowledge retard progress in solving these problems. *Escherichia coli* would be an ideal organism for studying these questions, but, until now, the basis of its substantial resistance to INH was unknown. A paradox posed by recent experiments (25), however, has provided a clue. Whereas expression of the *M. tuberculosis katG* gene correlated with INH susceptibility, its expression in *E. coli* did not result in susceptibility unless the *E. coli katG* gene was defective. Since *oxyR* (1) positively regulates the expression of *katG* (encoding hydroperoxidase I [HPI]), the *ahpC* and *ahpF* genes (encoding the two subunits of alkyl hydroperoxide reductase [AHP]), and six other genes in *E. coli* K-12, its role in INH resistance was examined. It is shown here that *oxyR* regulon mutants of *E. coli* and *Salmonella typhimurium* are INH susceptible.

Susceptibility of *E. coli* to INH was determined by (i) agar dilution and (ii) disk sensitivity tests. For agar dilution tests, cultures grown overnight in LB broth (15) at 37°C were diluted and plated on LB agar containing various concentrations of INH. After incubation for 24 h at 37°C, the efficiency of plating was determined by dividing the number of colonies on the plates with INH by the number on the control plates with no INH. For the disk sensitivity tests, 10 μl of the overnight cultures was plated in 2.5 ml of LB top agar (0.8% agar) on LB plates (1.2% agar). A 6.35-mm-diameter paper disk containing 10 μl of 3% cumene peroxide or $H_2O_2$ (19) or a 12.7-mm-diameter paper disk containing 25 μl of 0.91 M INH was then placed on the plate. The diameters of the zones of inhibition were measured after 24 h of incubation at 37°C. Data are the averages obtained from at least three experiments.

**INH-susceptible *E. coli*.** Whereas wild-type *E. coli* K-12 and an *oxyR* constitutive mutant (TA4110) were resistant to at least 500 μg of INH per ml, the *oxyR* null mutant GSO-8 was susceptible (Fig. 1; see Tables 1 and 2 for strain descriptions). The concentration of INH that inhibited colony formation by 50% ($IC_{50}$) was estimated to be 20 μg/ml. A strain in which *oxyR* had been deleted (TA4112) was similarly INH sensitive (Table 1, strains 1 to 5; data not shown). This suggests that OxyR- or OxyR-controlled functions are responsible for INH resistance in *E. coli*.

The roles of two OxyR-controlled functions were tested by transforming (11) GSO-8 with high-copy-number plasmids carrying *E. coli katG* or *ahp* genes (pBT22 [21] or pAQ27 [20], respectively) and determining the transformant's INH susceptibility. INH resistance was restored by the *ahp* plasmid but not by the parental plasmid pUC18 (23) or the pBR328-derived (17) *katG* plasmid (Fig. 1; Table 1, strains 6 to 8). Resistance to cumene peroxide and $H_2O_2$ is correlated with the expression of *ahp* and *katG* (4, 19), respectively. Table 1 also shows that susceptibility to cumene peroxide is correlated with INH susceptibility.

To determine whether AHP alone is responsible for high-level INH resistance in *E. coli*, the INH susceptibility of a mutant in which *ahp* had been deleted (TA4315) was tested

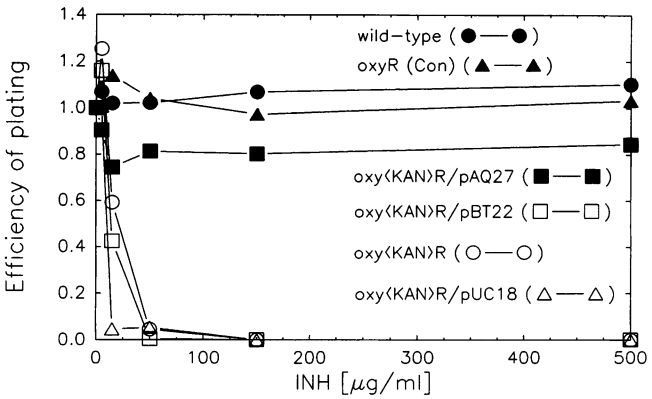


FIG. 1. Effects of an *oxyR* null mutation and overexpression of *ahp* on INH susceptibility. About 21% of the N7859 overnight culture was found to be ampicillin sensitive, indicating loss of the pAQ27 plasmid. Of the N7859 colonies appearing on plates with >15 μg of INH per ml, >95% were ampicillin resistant. Symbols: filled circles, K-12; filled triangles, TA4110; filled boxes, N7859; open circles, GSO-8; open triangles, N7883; open boxes, N7858.

* Electronic mail address: jlrosner@helix.nih.gov.

TABLE 1. Susceptibility of *E. coli* strains to INH, cumene peroxide, and $H_2O_2$

| Strain no. | Strain | Reference or source | Relevant genotype | Zone of inhibition diam (mm) | | |
|---|---|---|---|---|---|---|
| | | | | INH | Cumene peroxide | $H_2O_2$ |
| 1 | K-12 | 20 | Wild type | 13.1 | 16.4 | 18.2 |
| 2 | TA4110 | 20 | *oxyR2*(Con) | 12.7 | 12.7 | 14.1 |
| 3 | GSO-8 | G. Storz[a] | *oxy*<KAN>*R* | 32.1 | 33.1 | 44.0 |
| 4 | RK4936 | 20 | Wild type | 12.7 | 16.2 | 18.6 |
| 5 | TA4112 | 20 | *oxyR3*(Del) | 29.8 | 27.0 | 41.2 |
| 6 | N7883 | pUC18 × GSO-8 | *oxy*<KAN>*R*/pUC18 | 32.6 | 34.4 | 42.3 |
| 7 | N7858 | pBT22 × GSO-8 | *oxy*<KAN>*R*/pBT22(*katG*$^+$) | 26.5 | 32.5 | 25.0 |
| 8 | N7859 | pAQ27 (20) × GSO-8 | *oxy*<KAN>*R*/pAQ27(*ahp*$^+$) | 12.7 | 18.5 | 30.8 |
| 9 | TA4315 | 19 | *ahp5*(Del) | 23.1 | 32.2 | 18.6 |
| 10 | N7900 | P1 (14) grown on UM202 (19) × K-12 | *katG17*::Tn*10* | 12.7 | 15.0 | 26.8 |
| 11 | N7901 | P1 grown on UM202 × TA4315 | *katG17*::Tn*10 ahp5*(Del) | 33.0 | 34.9 | 44.3 |
| 12 | MP180 | 12 | Wild type | 12.7 | 18.3 | 19.9 |
| 13 | UM120 | 12 | *katE12*::Tn*10* | 12.7 | 17.7 | 20.2 |
| 14 | UM122 | 12 | *katF13*::Tn*10* | 13.1 | 18.3 | 22.1 |
| 15 | UM202 | 12 | *katG17*::Tn*10* | 13.2 | 14.9 | 32.3 |

[a] Strain GSO-8 was constructed by replacing *oxyR* sequences with a kanamycin-resistance cassette (*oxy*<KAN>*R*) (18).

(Fig. 2; Table 1, strain 9). TA4315 was more susceptible to INH ($IC_{50}$, ca. 175 μg/ml) than the wild type but not to the extent of that of the *oxyR*-inactivated strains. Since this suggested that another OxyR-controlled function was involved, the effect of inactivating *katG* in addition to *ahp* was evaluated (Fig. 2; Table 1, strains 10 and 11). While the *katG*::Tn*10* mutant (N7900) was barely more INH susceptible than the wild type, the *ahp katG* doubly mutant strain (N7901) approached GSO-8 in its INH susceptibility. Indeed, the efficiency of plating of N7901 on LB plates with 150 μg of INH per ml was $<10^{-7}$. Thus, AHP clearly contributes to INH resistance whereas the importance of the *katG*-encoded HPI becomes evident only when *ahp* is defective.

Other regulons that defend against oxidative stress include the *soxRS*-controlled superoxide response system (5, 22), the related *mar* (*soxQ*) multiple-antibiotic-resistance system (2, 3), and the *katF*-controlled stationary-phase adaptation system (8, 10). The effect of deleting the *soxRS* and/or *mar* (*soxQ*) region on INH susceptibility was examined by disk inhibition tests. No effect on INH susceptibility was observed (data not shown). Similarly, no increase in INH susceptibility was seen by inactivating either *katE*, encoding catalase HPII (9); *katF*, a positive regulator of *katE* and other genes; or *katG* (Table 1, strains 12 to 15). The effects of these regulons in a background in which *ahp* has been deleted have not been tested.

**INH-susceptible *S. typhimurium*.** Similar results were obtained with *S. typhimurium* LT2 strains (Table 2): (i) the *oxyR2* deletion rendered *S. typhimurium* INH susceptible (strains 1 and 2); (ii) the *ahp4* deletion resulted in partial INH susceptibility ($IC_{50}$, ca. 175 μg/ml) (strain 3 and data not shown); (iii) *oxyR*-independent expression of *ahp* by the *ahp-2* mutant partly reduced the INH susceptibility of the *oxyR2* mutant (strains 4 to 7); and (iv) the presence in the *ahp4* deletion strain of a multicopy plasmid (pAQ9) which carries the *S. typhimurium ahp* gene partly reduced its INH susceptibility (strain 8).

**Role of peroxidases in INH susceptibility.** These results suggest that AHP and HPI render INH inactive by destroying either peroxides needed to activate INH or the activated INH itself. Moreover, they indicate that interference with mycolic acid synthesis (13) is not the only growth-inhibiting action of INH since mycolic acid synthesis has not been identified in *E. coli*. Paradoxically, the work of Zhang et al. (24, 25) indicates that a KatG-like activity is necessary for INH susceptibility in *M. tuberculosis*, perhaps to render the INH active. One explanation is that the *M. tuberculosis* and *E. coli katG*-encoded catalase-peroxidases may differ in the relative activities of catalase to peroxidase. The *M. tuberculosis* KatG may have relatively more peroxidase function which activates INH while the *E. coli* (HPI) function may have more catalase activity which dissipates the peroxides needed to activate INH. However, because of the presence of AHP, HPI (katG) deficiency alone is insufficient to permit significant susceptibility in *E. coli*. This does not explain,

TABLE 2. Susceptibility of *S. typhimurium* strains to INH, cumene peroxide, and $H_2O_2$

| Strain no. | Strain | Reference | Relevant genotype | Zone of inhibition diam (mm) | | |
|---|---|---|---|---|---|---|
| | | | | INH | Cumene peroxide | $H_2O_2$ |
| 1 | LT2 | 20 | Wild type | 14.2 | 18.0 | 21.5 |
| 2 | TA4130 | 19 | *oxyR2*(Del) | 49.5 | 47.5 | 50.7 |
| 3 | TA4314 | 19 | *ahp4*(Del) | 24.5 | 31.2 | 22.7 |
| 4 | TT2385 | 1 | Wild type | 12.7 | 18.3 | 21.2 |
| 5 | TA4319 | 19 | *ahp-1*(Con) | 13.0 | 14.1 | 22.1 |
| 6 | TA4267 | 19 | *ahp-2*(Con) | 14.1 | 16.7 | 22.4 |
| 7 | TA4320 | 19 | *oxyR2*(Del) *ahp-2*(Con) | 24.3 | 20.2 | 33.8 |
| 8 | TA4317 | 19 | *ahp4*(Del)/pAQ9(*ahp*$^+$) | 18.8 | 13.0 | 27.0 |

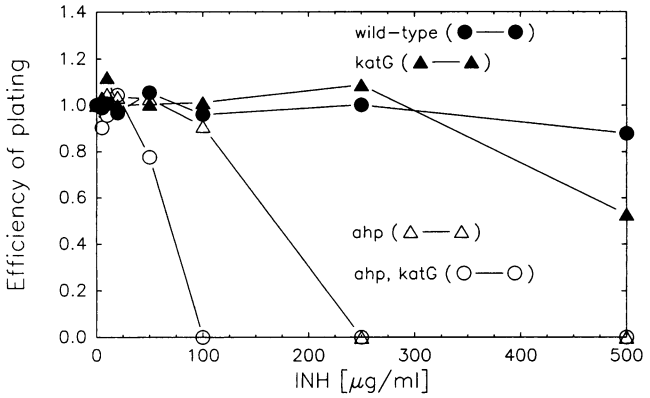


FIG. 2. Effects of *ahp* and/or *katG* genetic inactivation on INH susceptibility. Symbols: filled circles, K-12; filled triangles, N7900; open triangles, TA4315; open circles, N7901.

however, why *M. tuberculosis katG* can generate INH susceptibility in *E. coli* that is $Ahp^+$ (24). One possibility is that expression of *M. tuberculosis katG* on a multicopy plasmid represses *E. coli ahp* or inhibits AHP activity. Indeed, it may be that KatG has such a function even in *M. tuberculosis*. The recent report of the sequence of *M. tuberculosis katG* should promote investigation of this problem (6).

The mutants described here are about 1,000 times less susceptible to INH than is *M. tuberculosis* but are comparable in susceptibility to *M. smegmatis* (25). Nevertheless, by isolating INH-resistant mutants of N7901 or GSO-8, it may be possible to identify (i) INH-susceptible targets and (ii) functions (other than those of AHP and HPI) that normally contribute to INH resistance. Furthermore, these strains may already be sensitive enough to permit the selection of INH resistance genes from appropriate *E. coli* or mycobacterial libraries (7). Finally, these strains may be useful indicators of INH-like activity. By finding drugs to which these mutants (but not the wild type) are susceptible, the identification of new INH-like antibiotics may be facilitated.

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