A Novel Mechanism of Growth Phase-dependent Tolerance to Isoniazid in Mycobacteria*

Received for publication, December 13, 2011, and in revised form, May 27, 2012. Published, JBC Papers in Press, May 30, 2012, DOI 10.1074/jbc.M111.333385

Makoto Niki†, Mamiko Niki‡†, Yoshitaka Tateishi§§, Yuriko Ozeki††, Teruo Kirikae¶¶, Astrid Lewin**, Yusuke Inoue§§, Makoto Matsumoto†‡, John L. Dahl†††, Hisashi Ogura**, Kazuo Kobayashi†††, and Sohkichi Matsumoto‡‡‡

From the †Departments of Bacteriology and §§Virology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan, the ‡Department of Internal Medicine, National Hospital Organization Toneyama National Hospital, 5-1-1 Toneyama, Toyonaka, Osaka 560-8552, Japan, the ††Department of Food and Nutrition, Sonoda Women’s University, 7-29-1 Minamitsukaguchi-cho, Amagasaki, Hyogo 661-8520, Japan, the **National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan, the ***Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany, the ‡‡‡Microbiological Research Institute, Otsuka Pharmaceutical, Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan, the §§‡‡Department of Biology, University of Minnesota Duluth, Duluth, Minnesota 55812, and the §§‡Department of Immunology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Background: The mechanism underlying mycobacterial phenotypic tolerance to isoniazid is unknown.

Results: MDP1, a mycobacterial histone-like protein, down-regulates KatG expression.

Conclusion: Down-regulation of KatG by MDP1 causes growth phase-dependent phenotypic tolerance to isoniazid in mycobacteria.

Significance: Understanding the mechanism by which mycobacteria acquire tolerance to isoniazid is important for developing novel therapies.

Tuberculosis remains one of the most deadly infectious diseases worldwide and is a leading public health problem. Although isoniazid (INH) is a key drug for the treatment of tuberculosis, tolerance to INH necessitates prolonged treatment, which is a concern for effective tuberculosis chemotherapy. INH is a prodrug that is activated by the mycobacterial enzyme, KatG. Here, we show that mycobacterial DNA-binding protein 1 (MDP1), which is a histone-like protein conserved in mycobacteria, negatively regulates katG transcription and leads to phenotypic tolerance to INH in mycobacteria. Mycobacterium smegmatis deficient for MDP1 exhibited increased expression of KatG and showed enhanced INH activation compared with the wild-type strain. Expression of MDP1 was increased in the stationary phase and conferred growth phase-dependent tolerance to INH in M. smegmatis. Regulation of KatG expression is conserved between M. smegmatis and Mycobacterium tuberculosis complex. Artificial reduction of MDP1 in Mycobacterium bovis BCG was shown to lead to increased KatG expression and susceptibility to INH. These data suggest a mechanism by which phenotypic tolerance to INH is acquired in mycobacteria.

Tuberculosis is a disease caused by infection with Mycobacterium tuberculosis complex and remains a serious threat to health around the world. Approximately one-third of the world’s population is infected with M. tuberculosis. The current World Health Organization report shows that 8.8 million new tuberculosis cases arose, and 1.4 million people died from tuberculosis in 2010. Although medications are indispensable for treating infectious diseases, one of the predominant problems in tuberculosis chemotherapy is the prolonged treatment duration. The current treatment of tuberculosis with first-line antitubercular agents including isoniazid (isonicotinic acid hydrazide, INH)5, rifampin, pyrazinamide, streptomycin, and ethambutol requires at least six months to cure the acute disease, yet there is still a relapse rate of 2 to 3% (1). For latent tuberculosis, the standard treatment takes six to nine months using INH alone.

The relatively long duration of tuberculosis chemotherapy is not only due to reduced metabolism based on the slow growth rates of the pathogens but also the emergence of drug-resistant cells. There are two possible mechanisms by which M. tuberculosis acquires drug resistance. First, spontaneous chromosomal mutations in genes related to drug resistance can result from irregular drug supply, inappropriate drug prescriptions, and poor patient adherence to treatment (2). Secondly, M. tuberculosis can acquire phenotypic drug resistance in the absence of genotypic alterations in drug-target genes (3). In particular, it is well known that INH tolerance is acquired by M. tuberculosis

* This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology, the Ministry of Health, Labor, and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research Grants), The Japan Health Sciences Foundation, and The United States-Japan Cooperative Medical Science Program against Tuberculosis and Leprosy.

1 To whom correspondence may be addressed: Dept. of Bacteriology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. Tel.: 81-6-6645-3745; Fax: 81-6-6645-3746; E-mail: myoshih@med.osaka-cu.ac.jp.

2 To whom correspondence may be addressed: Dept. of Bacteriology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. Tel.: 81-6-6645-3745; Fax: 81-6-6645-3746; E-mail: sohkichi@med.osaka-cu.ac.jp.

3 The abbreviations used are: INH, isoniazid; BCG, Mycobacterium bovis bacillus Calmette-Guérin; MDP1, mycobacterial DNA-binding protein 1; MIC, minimum inhibitory concentration; NBT, Nitroblue tetrazolium; ADC, albumin-dextrose-catalase; qRT-PCR, quantitative RT-PCR; ANOVA, analysis of variance.
during the stationary phase, and requires prolonged tuberculosis chemotherapy.

INH is one of the key drugs used to control tuberculosis (4). It is critical in tuberculosis therapy because of its potent bactericidal activity against organisms growing actively in the pulmonary cavities, whereas the sterilizing activity of INH is reduced nearly 1,000-fold in *M. tuberculosis* cells during the stationary phase of growth (5).

The mode of action of this drug is complicated. INH is a prodrug that is converted into the active form by the mycobacterial catalase-peroxidase, KatG (6). The expression of KatG is regulated by an iron-containing transcription factor, *furA*, which is situated immediately upstream of *katG* (7). In its active form, INH inhibits both InhA, which is a primary target of INH (8), and an enoyl-acyl carrier protein reductase of the fatty acid synthase II (9, 10).

DNA sequencing of INH-resistant clinical isolates has revealed several mutations associated with resistance to INH. In addition to mutations in *katG* and *inhA*, *ahpC* (coding for alkyl hydroperoxide) (11) and *ndh* (coding for NADH dehydrogenase) (12) have also been reported to be associated with INH resistance. Mutations in *kasA*, which encodes ketoacyl acyl carrier protein synthase, are involved in INH resistance (13). However, later studies showed that overexpression of KasA did not lead to INH resistance in *M. tuberculosis* (14), and mutations in *kasA* are not likely to participate in INH resistance (4). Among these mechanisms, INH resistance in *M. tuberculosis* is most commonly associated with mutations in *katG*. In addition, a recent study by Ando *et al.* (15) revealed that mutations in the intergenic region of *furA* and *katG* also affected *katG* expression and conferred INH resistance. Although the reason why mycobacteria in the stationary phase acquire INH tolerance has not been fully elucidated, it is likely that the regulated expression of genes involved in the action of this drug is responsible for INH tolerance.

Transcriptional regulators are thought to play important roles in growth phase-dependent bacterial adaptive responses, including drug tolerance. Histone-like proteins are possible candidate transcriptional regulators in such responses. Recently, it was reported that Lsr2, a histone-like protein highly conserved in mycobacteria, inhibits a wide variety of DNA-interacting enzymes to regulate genes induced by antibiotics and those associated with inducible multidrug tolerance (16). Mycobacterial DNA-binding protein 1 (MDP1) is another histone-like protein in mycobacteria that binds to genomic DNA at guanine and cytosine residues. MDP1 is generally a negative regulator of gene expression (17) that participates in the slow growth rate of mycobacteria (18). Expression of this protein is enhanced in both stationary and dormant mycobacteria (19, 20).

In this study, we describe for the first time that MDP1 negatively regulates KatG expression, which, in turn, causes phenotypic tolerance to INH. The current study describes a novel molecular mechanism by which phenotypic drug tolerance is acquired in mycobacteria. This mechanism may strongly impact our understanding of phenotypic tolerance to INH in *M. tuberculosis*.  

### EXPERIMENTAL PROCEDURES

#### Bacterial Strains and Antimicrobial Agents—Mycobacterium smegmatis mc²155 (WT) and its histone-like protein/MDP1-deficient strain (MDP1-KO) were kindly provided by Dr. Thomas Dick (Novartis Institute for Tropical Diseases). An MDP1-complemented strain (MDP1-Comp) was generated previously (21). All *M. smegmatis* strains were cultured in Luria-Bertani (LB) broth or on LB agar plates (Sigma) aerobically at 37 °C on a magnetic stirrer set to rotate at 130 rpm. *Mycobacterium bovis* BCG strains were cultured in 7H9 broth base (Becton Dickinson and Company) supplemented with glycerol, 10% albumin-dextrose-catalase (ADC), and 0.05% Tween 80 (7H9-ADC-Tween), or on 7H11 agar plates supplemented with oleic acid, albumin, dextrose, and catalase (OADC). Ethambutol, rifampin, levofloxacin, and INH were purchased from Sigma. Rifampin was dissolved in ethanol, whereas levofloxacin, INH, and ethambutol were dissolved in distilled water. Stock solutions of each drug were filter-sterilized through 0.2-μm pore-size polyethersulfone membrane filters (Iwaki) except for rifampin, which was dissolved in ethanol.

#### Broth Microdilution—For the estimation of the minimum inhibitory concentrations (MICs) of each antibiotic, we used the broth microdilution method as previously described (22). Briefly, serial 2-fold dilutions of compounds were added to LB broth (for *M. smegmatis*) or 7H9-ADC-Tween (for BCG) to achieve final concentrations ranging from 128–0.125 μg/ml. The diluted antibiotic was then dispensed into the wells of microdilution plates at 0.1 ml per well. Aliquots of mycobacterial cells were then inoculated to a final concentration of ~10⁶ CFU/well. After incubation at 37 °C for 4 days (*M. smegmatis*) or 14 days (BCG), the MICs were determined as the lowest concentrations of compound that prevented visible growth.

#### Cell Viability against INH—*M. smegmatis* and BCG cells were grown aerobically at 37 °C in liquid medium under appropriate conditions for 2–6 days. At each time point, an aliquot of each culture was withdrawn and adjusted to an optical density at 600 nm (A₆₀₀) of 0.1 and subsequently diluted 1:100 in fresh medium. After addition of INH solution to a final concentration of 6.25 μg/ml (for *M. smegmatis*) or 0.125 μg/ml (for BCG), cells were grown aerobically at 37 °C for 24 h (*M. smegmatis*) or 48 h (BCG). Serial 10-fold dilutions of the cell suspensions were plated on agar plates to estimate the number of viable bacteria in the inoculum. After incubation for 4 days (*M. smegmatis*) or 14 days (BCG), the MICs were determined as the lowest concentrations of compound that prevented visible growth.

#### RNA Extraction—Cells were suspended in 1 ml of TRIzol reagent (Invitrogen) and disrupted using a Mini-BeadBeater. After incubation for 5 min at room temperature, 0.2 ml of chloroform was added, and the samples were shaken vigorously for 15 s. Cell lysates were centrifuged at 12,000 × g for 10 min at 4 °C, and the colorless upper aqueous phases were transferred to fresh tubes. Total RNA in the aqueous phase was precipitated by mixing samples with isopropyl alcohol followed by centrifugation. The pellets were washed with 75% ethanol, dried, and resuspended in 100 μl of diethylpyrocarbonate-treated dH₂O.
To remove the chromosomal DNA, samples were processed with a TURBO DNA-free kit (Applied Biosystems) according to the manufacturer’s instructions. RNA quantification was performed by spectrophotometry using a NanoDrop 3300 (Thermo Scientific).

**Microarray Analysis**—A customized high-density oligonucleotide whole genome expression array (NimbleGen Systems) was designed for *M. smegmatis mc²155* using the genome sequence and ORF predictions available from the J. Craig Venter Institute. Total RNA was extracted from *M. smegmatis* WT and MDP1-KO cells in the exponential phase (A<sub>600</sub> ~ 0.8). The cDNA synthesis, hybridization, and scanning were performed by NimbleGen Systems. Microarray data analysis was performed using GeneSpring GX (Agilent Technologies). The data presented are the results from one experiment.

**Real-time Quantitative RT-PCR (qRT-PCR)**—To confirm the results of microarray analysis, qRT-PCR was performed using Power SYBR green (Applied Biosystems). To generate cDNA samples, equal amounts of RNA were reverse transcribed using the High-capacity reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Primers were designed using Primer Express software (version 2.0; Applied Biosystems), and the sequences of the primers used were as follows: sigA (forward), 5'-CGTTCCCTCGACCTCATCCG-3'; sigA (reverse), 5'-GCCCTTGGTGTAGTCGAAC-TTC-3'; katG (forward), 5'-ACGCGCAATGACCTTTGCTG-3'; and katG (reverse), 5'-TGTCCGACTGGGCATAAACC-3'. A standard curve was generated for relative quantification of the PCR products, and a control reaction lacking reverse transcriptase was performed for every RNA sample. The same amount of total protein from each strain (2 μg per well) was separated by SDS-PAGE. Immunoblotting was carried out as described previously (26, 27). Anti-heat shock protein Hsp65 (GroEL2) antibody was used as a loading control (26, 27). The expression levels of different proteins were analyzed using the public domain software Image J (a Java image processing program developed by the National Institutes of Health Image for Macintosh).

**Reduction of Nitroblue Tetrazolium (NBT)**—This test detects INH activation by KatG and is dependent on the reduction of NBT by superoxide-free radical in the presence of INH to form an insoluble formazan. Reduction was monitored qualitatively following electrophoresis of whole-cell extracts on native gels, as described previously (28). Before samples were subjected to native PAGE, protein concentrations were quantified by Bradford assay. The gel was soaked in 50 ml of 50 mM sodium phosphate (pH 7.0) containing 68 mg of INH, 12.5 mg of NBT, and 15 ml of 30% H<sub>2</sub>O<sub>2</sub>. Color development was complete after 30 min. The gel was then rinsed with distilled water and soaked in 7% acetic acid, 1% glycerol in distilled water before visualization. The levels of KatG activity in each strain observed in the native PAGE gel were also analyzed using ImageJ software.

**Transformation and Isolation of Recombinant M. bovis BCG Strains**—Preparation of *M. bovis* BCG competent cells and electroporation procedures were performed according to standard procedures (29). Briefly, *M. bovis* BCG strain Tokyo was grown in 7H9-OADC-Tween until an A<sub>600</sub> of 0.6 was reached. Cells were harvested by centrifugation, washed several times, and resuspended in one-tenth of their original volume of 10% glycerol to obtain competent cells. The MDP1-antisense plasmid (18) as well as the empty vector, pMV261, were introduced into BCG-competent cells using a Gene pulser II (Bio-Rad) with the following settings: 2.5 kV, 129 ohms, 50 μF. After electroporation, cells were resuspended in 4 ml of 7H9-ADC-Tween and incubated for 20 h at 37 °C. Following incubation, appropriate dilutions were added to 7H11 agar containing 10 μg/ml kanamycin. One clone of empty vector transformant (pMV261) and two clones of transformants carrying MDP1-antisense plasmid (pAS-MDP1-1 and pAS-MDP1-2) were chosen for further analysis.

**Expression and Purification of Recombinant MDP1**—Recombinant MDP1 was obtained as described previously (30). Briefly, the oligonucleotide primers (forward, 5'-CCCCCATATGAAACAGCAGCTCATTTGAC-3'; reverse, 5'-CCCCAACGT- TTTCGCGACCCTGCCGAGCCG-3') were synthesized based on the nucleotide sequences of BCG and *M. tuberculosis* mdp1. The amplified DNA fragment were digested with Ndel and HindIII, cloned into the same sites of pET22b (Novagen, Darmstadt, Germany), and introduced into *Escherichia coli* BL21 (DE3). Expression of recombinant protein was induced by addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were then incubated for 10 h at 32 °C. After incubation, cells were disrupted, and the supernatant was collected by centrifugation 8,000 × g for 30 min. After filtering the supernatant through a 0.22-μm Hi-Trap column charged previously with 100 mM NiSO<sub>4</sub> and equilibrated with 20 mM sodium phosphate, pH 7.4, 10 mM imidazole, and 0.5 M NaCl. After washing out unbound proteins, the protein was eluted with the same buffer containing 300 mM imidazole. The fractions containing MDP1 were collected and dialyzed against PBS. The purity of the protein was confirmed by staining as a single band with Coomassie Brilliant Blue R-250 following separation by SDS-PAGE.

**Electrophoretic Mobility Shift Assay (EMSA)**—Primer (forward, 5'-CTCTGACAGGCCGCAATGCG-3'; reverse, 5'-GAC- CAGAGGCCTACTGTTTT-3') were used to amplify a 80-bp fragment containing the furA promoter region, as described by Milano et al. (31). The amplified DNA fragment was labeled with digoxigenin with a digoxigenin gel shift kit, second generation (Roche Diagnostics), according to the manufacturer’s protocol. EMSA was also performed using the same kit according to the manufacturer’s instructions. Briefly, purified recombinant MDP1 protein was incubated with 40 fg of digoxigenin-labeled double-stranded DNA fragments in a final volume of 25 μl. Incubations were carried out at 4 °C for 2 h in a solution of 10
Isoniazid Tolerance in Mycobacteria

\[ \text{MIC of MDP1-KO cells was 8.0 \mu g/ml, whereas the MICs for the other strains were 4-fold higher. In contrast, } M. \text{ smegmatis WT, MDP1-KO, and MDP1-Comp were equally susceptible to the other drugs tested, including ethambutol, rifampin, and levofloxacin (Table 1).} \]

To clarify the effects of culture medium components on susceptibility to antibiotics, we performed broth microdilution assays using 7H9-ADC-Tween as the culture medium. The MICs of each strain in 7H9-ADC-Tween were the same as those in LB broth (data not shown). To confirm the differential susceptibility to INH between strains, we counted the CFUs in each strain after exposure to INH at a concentration below the MIC for MDP1-KO cells (Fig. 1B). We found that MDP1-KO cells showed decreased viability following treatment with INH compared with WT and MDP1-Comp cells. These results suggested that susceptibility to INH is affected by the presence of MDP1 in M. smegmatis cells. Our results are consistent with the data recently reported by Dahl et al. (33).

Increased Transcription of \textit{katG} in MDP1-KO—To investigate the effect of MDP1 on the expression of genes related to INH resistance, we performed DNA microarray analysis to compare gene expression profiles between M. smegmatis WT and MDP1-KO strains. The expression levels of the genes related to INH resistance, such as \textit{inhA} and \textit{ahpC}, were similar in MDP1-KO and WT cells (Table 2). However, a significant increase in \textit{katG} expression was observed in MDP1-KO cells.

To confirm the increased \textit{katG} transcription in MDP1-KO cells, total RNA from M. smegmatis WT, MDP1-KO, and MDP1-Comp cells was isolated and used for qRT-PCR analysis of \textit{katG} expression. The expression levels of \textit{katG} mRNA were determined by the comparative threshold cycle method and then normalized to \textit{sigA} expression. As expected, MDP1-KO cells possessed a 2-fold higher expression level of \textit{katG} mRNA compared with WT cells (Fig. 2). In contrast, MDP1-Comp cells exhibited 2-fold lower \textit{katG} expression than the WT strain, whereas the viability of MDP1-Comp cells treated with INH was similar to that of WT cells.

Increased KatG Protein Expression in MDP1-KO—To support the results of DNA microarrays and qRT-PCR, Western blotting was used to analyze the expression level of KatG protein. As a loading control, anti-Hsp65 antibody was used to confirm that the protein concentrations were equal in all samples. The results demonstrated that M. smegmatis WT and MDP1-Comp cells produced similar amounts of KatG, whereas KatG expression was increased in MDP1-KO cells (Fig. 3A).

**TABLE 1**

| MICs against various antibiotics by the broth microdilution method | EB | RFP | LVFX | INH |
|-------------------------|----|-----|------|-----|
| WT         | 1.0 | 8   | 0.5  | 32  |
| KO         | 1.0 | 8   | 0.5  | 8   |
| Comp       | 1.0 | 8   | 0.5  | 8   |

**TABLE 2**

Expression of genes related to INH resistance analyzed by microarray

|                      | WT      | MDP1-KO |
|----------------------|---------|---------|
| MSMEG_3461 (katG)    | 2.910772| 6.552576|
| MSMEG_3515 (inhA)    | 3.056271| 2.722855|
| MSMEG_4891 (ahpC)    | 164.84808| 168.63435|

---

**RESULTS**

MDP1-deficient M. smegmatis Showed Increased Susceptibility to INH—To determine whether MDP1 is involved in the acquisition of drug sensitivity in mycobacteria, we analyzed the susceptibility of M. smegmatis WT, MDP1-KO, and MDP1-Comp cells to antibiotics. As shown in Fig. 1A, a marked increase in susceptibility to INH was observed in MDP1-KO cells. We determined the MICs of INH for each strain using the broth microdilution method described previously (32). As presented in Table 1, the MIC of MDP1-KO cells was 8.0 \mu g/ml,
These data suggest that MDP1 regulates the KatG protein level in *M. smegmatis* cells.

**Enhanced Activation of INH in MDP1-KO Cells**—Next, we investigated whether INH activation is increased in MDP1-KO cells, which produce larger amounts of KatG than WT cells (Fig. 3A). The INH-dependent NBT reduction assay is commonly used to estimate the level of INH activation by KatG because the KatG enzyme produces free radicals upon activation of INH (34, 35).

The NBT reduction assay was performed using cell lysates derived from *M. smegmatis* WT, MDP1-KO, and MDP1-Comp cells. A single band of activity was detected in each lane at the same distance on the gel as a purple formazan product. As shown in Fig. 3B, whole-cell lysates of MDP1-KO cells exhibited a higher capacity to reduce NBT to formazan in the presence of INH than other strains. To confirm that the different NBT-reducing activities between strains were due to different KatG expression levels in each strain and not to amino acid substitutions, we compared their *katG* nucleotide sequences and found no amino acid substitutions (data not shown). These data suggest that increased expression of KatG results in the activation of INH, and consequently, MDP1-KO cells are less viable following treatment with INH.

**MDP1 Affects INH Susceptibility in Growth Phase-dependent Manner**—Our previous study revealed that the expression of MDP1 is up-regulated in the stationary phase of mycobacterial culture (21). Therefore, we investigated whether the different expression levels of MDP1 during different growth states produced the INH-resistant phenotype in *M. smegmatis*. The expression of MDP1 was shown to increase in both WT and MDP1-Comp cells during the stationary growth phase (day 6 in Fig. 4). In contrast, expression of KatG was observed during the...
logarithmic growth phase in both WT and MDP1-Comp cells but diminished during the stationary growth phase (Fig. 4). MDP1-KO cells, however, showed enhanced KatG expression during the logarithmic growth phase (Fig. 2), whereas KatG was still expressed even in the stationary growth phase (Fig. 4).

A time course analysis was performed investigating the expression levels of MDP1 and KatG and the INH susceptibility of *M. smegmatis* WT cells. Western blotting revealed a time-dependent up-regulation of MDP1 expression, whereas the expression of KatG was inversely decreased (Fig. 5A). Cell viability in the presence of INH was also found to vary at each time point. On day 2 (exponential phase), a significant reduction in the number of CFUs was observed after treatment with INH at a concentration of 0.0265 μg/ml or 6.25 μg/ml for 2 days. After INH treatment, the drug was removed from the culture media, and cells were diluted and plated onto INH-free LB agar plates. The number of viable cells obtained following treatment with INH (open bar) was compared with the number obtained without INH treatment (solid bar) at each time point. Mean values and S.E. for three experiments are shown. The statistical significance of the differences in CFUs between the different time points was determined by one-way ANOVA. *, p < 0.01.

*M. bovis* BCG clones carrying the MDP1-antisense plasmid (pAS-MDP1-1 and pAS-MDP1-2) and the reference strain (pMV261). *M. bovis* BCG is an attenuated strain of *M. bovis* that belongs to the *M. tuberculosis* complex. The architecture of the katG gene and its upstream regulatory region are the same in *M. bovis* BCG and *M. tuberculosis*. Western blotting showed that MDP1 expression was decreased by half, and KatG expression was increased by 2-fold, in both pAS-MDP1-1 and pAS-MDP1-2 compared with pMV261 (Fig. 6A). We then determined the MICs of these strains in the presence of INH and found that mutants with reduced MDP1 expression showed increased susceptibility to INH, consistent with data obtained for *M. smegmatis* MDP1-KO (Figs. 6 and 7). These results suggest that MDP1 influences the susceptibility of not only the avirulent rapidly growing *M. tuberculosis* complex but also the slow growing *M. tuberculosis* complex to INH.

**MDP1 Binds to DNA Sequence in FurA-KatG Promoter Region**—A previous report revealed that *katG* is negatively regulated by FurA, a homologue of the ferric uptake regulator Fur, by binding to its promoter region (36). It was also shown that
**furA** and **katG** are co-transcribed from a common regulatory region located immediately upstream of the **furA** gene (31). Furthermore, inactivation of **furA** resulted in **katG** up-regulation, which increased the sensitivity of mycobacterial cells to INH (7). We hypothesized that MDP1 may directly bind to the promoter region of the FurA-KatG operon and performed gel-shift assays to test the hypothesis. We observed that co-incubation of MDP1 with a digoxigenin-labeled DNA fragment containing the **furA**-**katG** promoter region resulted in the disappearance of a DNA band. This was due to altered electrophoretic mobility of the MDP1-DNA complex because of the high basic charge of MDP1 (pI_12.4). We confirmed the specificity of this binding by showing the absence of a band shift in the presence of non-digoxigenin-labeled competitor (Fig. 8). These data show that MDP1 has the ability to bind to the **furA**-**katG** promoter region.

**DISCUSSION**

MDP1 is a conserved histone-like DNA binding protein found in mycobacteria including *M. tuberculosis*, *Mycobacterium leprae*, and *M. smegmatis*. Because chromatin-associated proteins are thought to organize the bacterial chromosome and exert a regulatory influence on transcription, recombination and DNA replication (37), it has been postulated that MDP1 may contribute to the regulation of gene expression. In this study, we found that MDP1 down-regulates the expression of KatG in mycobacteria.

KatG is an antioxidant enzyme that converts hydrogen peroxide (H₂O₂) into water and oxygen via its catalase activity (38, 39). Because mycobacteria encounter oxidative stress inside host cells, antioxidant proteins such as KatG play a significant role in intracellular survival. Sherman *et al.* (40) reported that INH-resistant katG mutants acquired a second mutation...
resulting in hyperexpression of alkyl hydroperoxidase (AhpC) to compensate for the loss of KatG activity in the detoxification of organic peroxides. However, overexpression of AhpC did not result in increased M. tuberculosis survival, and deletion of ahpC did not alter the expression of katG. Thus, AhpC does not compensate for the loss of KatG activity. In addition to DNA-binding activity, we discovered recently that MDPI enzymatically converts H$_2$O$_2$ into water and oxygen in the presence of Fe$^{2+}$ (41). Therefore, it is conceivable that both KatG and MDPI play a significant role in H$_2$O$_2$ detoxification in mycobacterial cells. MDPI rather than AhpC may compensate for reduced KatG activity, and therefore their expression levels are reciprocally regulated.

It is also known that katG is regulated negatively by FurA, a homologue of the ferric uptake regulator Fur, which is encoded by a gene located immediately upstream of katG (36). Similar to FurA, MDPI has affinity to iron (41), and the expression levels of both FurA and MDPI are influenced by the environmental iron concentration (42). EMSA assay revealed that MDPI has an actual capacity to bind to the promoter region of furA-katG, suggesting that MDPI is a trans-acting factor that regulates the transcription of the FurA-KatG operon by directly binding to its promoter region. Future studies, including chromatin immunoprecipitation assays, will clarify the effect of MDPI on its promoter region. Thus, cells with reduced KatG activity may be phenotypically tolerant to INH.

Drug tolerance is a phenomenon seen in many microorganisms during their growth in vivo (3, 43, 44). It is known that phenotypic tolerance occurs when the environmental or physiological status of the bacteria change. For example, environmental factors such as low pH (45), depletion of certain nutrients (46), and high magnesium or calcium concentration (47) induce phenotypic tolerance. The inhibition of growth occurs in the stationary phase and is the most common cause of reduced drug susceptibility in all bacteria (48).

It has been reported that the expression of histone-like proteins varies with the age of the culture. For example, Fis is a cofactor in a site-specific recombination system that is expressed at a high level in the early exponential phase of E. coli growth (49). In contrast, integration host factor is found to be most abundant when the culture enters the stationary phase (50). Therefore, the expression of MDPI is enhanced in the stationary growth phase (20), this molecule may affect the gene profile of cells in the stationary phase. The current study shows that alteration of MDPI expression induces growth phase-dependent tolerance to INH by controlling KatG expression in M. smegmatis. A schematic diagram showing a hypothetical mechanism of INH tolerance regulated by MDPI is provided in Fig. 9. To our knowledge, this is the first description of a molecular mechanism underlying growth phase-dependent tolerance to INH in mycobacteria.

Regulation of KatG expression appears to be conserved in several mycobacteria, including M. tuberculosis and M. smegmatis (31). We show here that reduction of MDPI expression by antisense knockdown increases the sensitivity of M. bovis BCG to INH (Fig. 7). M. bovis BCG and M. tuberculosis show similar susceptibilities to INH, and the DNA sequences of their katG genes and upstream regulatory regions are identical. Taken together, MDPI may affect the expression level of KatG and the susceptibility of pathogenic M. tuberculosis to INH. Our data suggest that the artificial reduction of MDPI expression may enhance the efficacy of INH and shorten the length of tuberculosis chemotherapy against both active and latent diseases.

**Acknowledgments**—We are grateful to Dr. Thomas Dick (Novartis Institute for Tropical Diseases) for providing Ms-MDP1/HLP-KO M. smegmatis. We also thank Sara Matsumoto and Chihiro Inoue for assistance with the experiments and encouragement.

**REFERENCES**

1. Ormerod, L. P. (2008) The evidence based treatment of tuberculosis: Where and why are we failing? *Thorax* 63, 388–390
2. Nachega, J. B., and Chaisson, R. E. (2003) Tuberculosis drug resistance: A global threat. *Clin. Infect. Dis.* 36, S24–30
3. Wallis, R. S., Patil, S., Cheon, S. H., Edmonds, K., Phillips, M., Perkins, M. D., Joloba, M., Namale, A., Johnson, J. L., Teixeira, L., Dietze, R., Siddiqi, S., Mugerwa, R. D., Eisenach, K., and Ellner, J. I. (1999) Drug tolerance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 43, 2600–2606
4. Vilchèze, C., and Jacobs, W. R., Jr. (2007) The mechanism of isoniazid killing: Clarity through the scope of genetics. *Annu. Rev. Microbiol.* 61, 35–50
5. Herbert, D., Paramasivan, C. N., Venkatesan, P., Kubendiran, G., Prabhakar, R., and Mitchison, D. A. (1996) Bactericidal action of ofloxacin, sulfactam-ampicillin, rifampin, and isoniazid on logarithmic- and stationary-phase cultures of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 40, 2296–2299
6. Zhang, Y., Heym, B., Allen, B., Young, D., and Cole, S. (1992) The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* 358, 591–593
7. Pym, A. S., Domenech, P., Honoré, N., Song, J., Deretic, Y., and Cole, S. T. (2001) Regulation of catalase-peroxidase (KatG) expression, isoniazid sensitivity, and virulence by furA of *Mycobacterium tuberculosis*. *Mol. Microbiol.* 40, 879–889
8. Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., Collins, D., de Lisle, G., and Jacobs, W. R., Jr. (1994) inha, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* 263, 227–230
9. Dessen, A., Quémard, A., Blanchard, J. S., Jacobs, W. R., Jr., and Sacchet-
tini, J. C. (1995) Crystal structure and function of the isoniazid target of
Mycobacterium tuberculosis. Science 267, 1638–1641
10. Quémard, A., Sacchettni, J. C., Dessen, A., Vilchère, C., Bittman, R., Ja-
cobs, W. R., Jr., and Blanchard, J. S. (1995) Enzymatic characterization of
the target for isoniazid in Mycobacterium tuberculosis. Biochemistry 34,
8235–8241
11. Wilson, T. M., and Collins, D. M. (1996) ahpC, a gene involved in isoniazid
resistance of the Mycobacterium tuberculosis complex. Mol. Microbiol. 19,
1025–1034
12. Miesel, L., Weisbrod, T. R., Marcinkeviciene, J. A., Bittman, R., and Jacobs,
W. R., Jr. (1998) NADH dehydrogenase defects confer isoniazid resistance
and conditional lethality in Mycobacterium smegmatis. J. Bacteriol. 180,
2459–2467
13. Colangelini, R., Helb, D., Vilchère, C., Hazbón, M. H., Lee, C. G., Safi, H.,
Sayers, B., Sardone, I., Jones, M. B., Fleischmann, R. D., Peterson, S. N.,
Jacobs, W. R., Jr., and Alland, D. (2007) Transcriptional regulation of multi-
drug tolerance and antibiotic-induced responses by the histone-like protein
Lsr2 in M. tuberculosis. PLoS Pathog. 3, e87
14. Matsumoto, S., Furugen, M., Yukitake, H., and Yamada, T. (2000) The gene
encoding mycobacterial DNA-binding protein 1 (MDP1) transformed
rapidly growing bacteria to slowly growing bacteria. FEMS Microbiol.
Lett. 182, 297–301
15. Lewin, D., Baus, D., Kamel, E., Bon, F., Kunisch, R., Maurischat, S., Adono-
poulou, M., and Eich, K. (2008) The mycobacterial DNA-binding protein
1 (MDP1) from Mycobacterium bovis BCG influences various growth
characteristics. BMC Microbiol. 8, 91
16. Dick, T., Lee, B. H., and Murugasu-Oei, B. (1998) Oxygen depletion-in-
duced dormancy in Mycobacterium smegmatis. FEMS Microbiol. Lett.
163, 159–164
17. Matsumoto, S., Yukitake, H., Furugen, M., Matsuo, T., Mineta, T., and
Yamada, T. (1999) Identification of a novel DNA-binding protein from
Mycobacterium bovis Bacillus Calmette-Guérin. Microbiol. Immunol. 43,
1027–1036
18. Katsube, T., Matsumoto, S., Takatsuka, M., Okuyama, M., Ozeki, Y.,
Naito, M., Nishiiishi, Y., Fujisawa, N., Yoshimura, M., Tsuoi, T., Torii,
M., Oshitani, N., Arakawa, T., and Kobayashi, K. (2007) Control of cell
wall assembly by a histone-like protein in Mycobacteria. J. Bacteriol. 189,
8241–8249
19. Brown, B. A., Wallace, R. J., Jr., and Onyi, G. O. (1992) Activities of clari-
thromycin against eight slowly growing species of nontuberculous myco-
bacteria, determined by using a broth microtitration MIC system. Antimi-
crob. Agents Chemother. 36, 1987–1990
20. Yuan, Y., Crane, D. D., Simpson, D., Zhu, Y. Q., Hickey, M. J., Sherman,
D. R., and Barry, C. E., 3rd (1998) The 16-kDa α-crystallin (AcR) protein of
Mycobacterium tuberculosis is required for growth in macrophages. Proc.
Natl. Acad. Sci. U.S.A. 95, 9578–9583
21. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation
of microgram quantities of protein utilizing the principle of protein-dye
binding. Anal. Biochem. 72, 248–254
22. Sekiguchi, J., Miyoshi-Akiyama, T., Augustynowicz-Kopec, E., Zwołska,
Z., Kirikae, F., Toyota, E., Kobayashi, I., Morita, K., Kudo, K., Kato,
S., Kuratsuji, T., Mori, T., and Kirikae, T. (2007) Detection of multidrug
resistance in Mycobacterium tuberculosis. J. Clin. Microbiol. 45, 179–192
23. Blasco, B., Chen, J. M., Hartkoom, R., Sala, C., Uplekar, S., Rougemont, J.,
Pojer, F., and Cole, S. T. (2012) Virulence regulator EspR of Mycobac-
teriaceae, an antituberculosis drug target. PLoS Pathog. 8, e1002621
24. Horne, D., and Tomasz, A. (1981) pH-dependent penicillin tolerance of
Mycobacterium smegmatis. J. Bacteriol. 149, 642–646
25. Smith, A. L., Fiel, S. B., Mayer-Hamblett, N., Ramsey, B., and Burns, J. L.
(2003) Susceptibility testing of Pseudomonas aeruginosa isolates and clinical
response to parenteral antibiotic administration: Lack of association in
cystic fibrosis. Chest 123, 1495–1502
26. Horne, D., and Tomasz, A. (1981) pH-dependent penicillin tolerance of
Mycobacterium smegmatis. J. Bacteriol. 149, 642–646
27. Smith, A. L., Fiel, S. B., Mayer-Hamblett, N., Ramsey, B., and Burns, J. L.
(2003) Susceptibility testing of Pseudomonas aeruginosa isolates and clinical
response to parenteral antibiotic administration: Lack of association in
cystic fibrosis. Chest 123, 1495–1502
28. Horne, D., and Tomasz, A. (1981) pH-dependent penicillin tolerance of
Mycobacterium smegmatis. J. Bacteriol. 149, 642–646
29. Smith, A. L., Fiel, S. B., Mayer-Hamblett, N., Ramsey, B., and Burns, J. L.
(2003) Susceptibility testing of Pseudomonas aeruginosa isolates and clinical
response to parenteral antibiotic administration: Lack of association in
cystic fibrosis. Chest 123, 1495–1502
30. Horne, D., and Tomasz, A. (1981) pH-dependent penicillin tolerance of
Mycobacterium smegmatis. J. Bacteriol. 149, 642–646
46. Kim, K. S., and Bayer, A. S. (1987) Significance of in vitro penicillin tolerance in experimental enterococcal endocarditis. J. Antimicrob. Chemother. 19, 475–485
47. Goodell, E. W., Fazio, M., and Tomasz, A. (1978) Effect of benzylpenicillin on the synthesis and structure of the cell envelope of Neisseria gonorrhoeae. Antimicrob. Agents Chemother. 13, 514–526
48. Tuomanen, E. (1986) Phenotypic tolerance: The search for β-lactam antibiotics that kill nongrowing bacteria. Rev. Infect. Dis. 8, S279–291
49. Bradley, M. D., Beach, M. B., de Koning, A. P., Pratt, T. S., and Osuna, R. (2007) Effects of Fis on Escherichia coli gene expression during different growth stages. Microbiology 153, 2922–2940
50. Ali Azam, T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. (1999) Growth phase-dependent variation in protein composition of the Escherichia coli nucleoid. J. Bacteriol. 181, 6361–6370