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katGI and *katGII* Encode Two Different Catalases-Peroxidases in *Mycobacterium fortuitum*

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It has been suggested that catalase-peroxidase plays an important role in several aspects of mycobacterial metabolism and is a virulence factor in the main pathogenic mycobacteria. In this investigation, we studied genes encoding for this protein in the fast-growing opportunistic pathogen *Mycobacterium fortuitum*. Nucleotide sequences of two different catalase-peroxidase genes (*katGI* and *katGII*) of *M. fortuitum* are described. They show only 64% homology at the nucleotide level and 55% identity at the amino acid level, and they are more similar to catalases-peroxidases from different bacteria, including mycobacteria, than to each other. Both proteins were found to be expressed in actively growing *M. fortuitum*, and both could also be expressed when transformed into *Escherichia coli* and *M. aurum*. We detected the presence of a copy of IS*6100* in the neighboring region of a *katG* gene in the *M. fortuitum* strain in which this element was identified (strain FC1). The influence of each *katG* gene on isoniazid (isonicotinic acid hydrazide; INH) susceptibility of mycobacteria was checked by using the INH-sensitive *M. aurum* as the host. Resistance to INH was induced when *katGI* was transformed into INH-sensitive *M. aurum*, suggesting that this enzyme contributes to the natural resistance of *M. fortuitum* to the drug. This is the first report showing two different genes encoding same enzyme activity which are actively expressed within the same mycobacterial strain.

Mycobacterium is an old bacterial genus which includes species from a broad range of ecological niches (14). Pathogenicity ranges from strict specificity for humans or animals, such as *Mycobacterium tuberculosis*, *M. leprae*, or *M. paratuberculosis*, to specificity for saprophytes, such as *M. smegmatis* and *M. phlei*. Occupying an intermediate position is *M. fortuitum*, which can be isolated from clinical samples as an opportunistic pathogen and from environmental sources as a saprophyte (38).

The level of adaptation that bacteria have to their environment is possibly related to the number of genes that they are able to activate as a consequence of detection of changes in its environment. The genomic length of the intracellular restricted mycobacterium *M. leprae* is smaller than that of other mycobacteria (6, 11, 28). This possibly indicates that *M. leprae* requires fewer genes for survival within its host due to the stability of its environment.

Mycobacteria are considered archetypical intracellular pathogens because of their capacity to invade and multiply within macrophages, and they have evolved mechanisms for evading macrophage-mediated killing. An important mechanism employed by macrophages is the production of toxic reactive oxygen intermediates (5, 27). Catalases-peroxidases are enzymes which are used by bacteria to protect themselves against reactive oxygen intermediates, and hence they are likely to be important for survival within macrophages (27).

Catalase-peroxidase plays a crucial enzymatic role in mycobacterial metabolism by increasing the chance of survival inside macrophages. This is achieved by reducing toxic forms of oxygen formed as a consequence of the respiratory burst in phagocytic cells and by inhibiting the nitric oxide synthesis related to the oxygen-independent antimicrobial activity (27).

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Isoniazid (isonicotinic acid hydrazide; INH) is one of the major drugs currently used in the chemotherapy of tuberculosis. Catalase-peroxidase also activates INH to a toxic oxidated derivative, thereby inhibiting the biosynthesis of cell wall mycolic acids by linkage to other enzymes from the mycolic acids biosynthetic pathway (29). Although several genes (*inhA*, *katG*, and *oxyR*) are involved in the mechanism of INH resistance in mycobacteria, the development of resistance in *M. tuberculosis* is often associated with a loss of catalase-peroxidase activity through mutations in the sequence of its *katG* gene (1, 17).

Mycobacteria can produce two classes of catalases: the heat-labile or T-catalase, which has also a peroxidase-like function (similar to *Escherichia coli* HPI), and the heat-stable or M-catalase (similar to *E. coli* HPII) (35). Some species of mycobacteria produce only one class of catalase (T in *M. tuberculosis* or M in *M. terrae*), and other species, e.g., the majority of fast-growing mycobacteria (2, 10, 36), produce both.

Genes encoding catalase-peroxidase (*katG*) from slow-growing mycobacteria, such as *M. tuberculosis* (16) and *M. intracellulare* (24), have been cloned and sequenced. Polymorphism associated with the *katG* region has been reported for *M. tuberculosis*. The *katG* gene seems to be located in a particularly variable region within the *M. tuberculosis* chromosome. This characteristic may favor *katG* mutations occurring, with consequent changes in INH strain sensitivity (1, 17, 45).

The normal presence of the M-catalase activity in INH-resistant mycobacteria was previously considered to be involved in antagonizing T-catalase action on INH in resistant mycobacteria; therefore, organisms having both M- and T-catalases were thought to be constitutively resistant to INH at different levels. On the other hand, it has been recently observed that M-catalase did not show any antagonistic effect on INH activity in *M. avium* (23).

Other genes have been described as being related to INH sensitivity in mycobacteria. *inhA* and *oxyR* particularly have been suggested to be involved in this resistance. *inhA* encodes for the enzyme enoyl-acyl carrier protein reductase, which is

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Source or reference[b]
Strain[a]		
M. avium 26C		H.12 Octubre
M. aurum L1		15
+pRMFR	Kan^r	This report
+pRMFP	Kan^r	This report
+pRR3	Amp^r Kan^r	This report
M. fortuitum ATCC 6841^T		
M. fortuitum FC1		19
M. fortuitum ATCC 13756^T		
M. fortuitum 1250		CNM
M. intracellulare		H.12 Octubre
M. smegmatis CIPT 141330010^T		
M. tuberculosis 79500	INH sensitive	44
M. tuberculosis B1453	INH resistant, *katG*	44
E. coli DH5α	Φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*	Laboratory strain
+pMFR	Amp^r	This report
+pMFP	Amp^r	This report
+pIPC17	Amp^r	This report
Plasmid		
pUC18	Amp^r	40
pRR3	Amp^r Kan^r	30
pIPC17	Amp^r	19
pMFR	pUC18 construct containing a 4.3-kbp *Eco*RI insert including *katGI* gene	This report
pMFP	pUC18 construct containing a 4.5-kbp *Sac*I insert including *katGII* gene	This report
pRMFR	pRR3 construct containing a 4.3-kbp *Eco*RI insert including *katGI* gene	This report
pRMFP	pRR3 construct containing a 4.5-kbp *Sac*I insert including *katGII* gene	This report

[a] CIPT, Collection Institut Pasteur Tuberculose; ATCC, American Type Culture Collection.
[b] H.12 Octubre, Hospital 12 de Octubre (E. Palenque); CNM, Centro Nacional de Microbiologia, Majadahonda, Madrid, Spain.

part of the mycolic acid biosynthetic pathway (29), and the *oxyR* gene product controls expression of several other genes, including *katG*. This last regulatory protein is inactive in members of the *M. tuberculosis* complex but active in other INH-resistant mycobacteria (7, 8).

The majority of human infections attributed to fast-growing mycobacteria are caused by members of the *Mycobacterium fortuitum* complex (38). *M. fortuitum* in particular has been described as a human pathogen (26). It has often been chosen as a model for studying the intracellular killing of mycobacteria (13, 26), and its gene encoding superoxide dismutase (*sodA*) has also been studied (22). It is relatively resistant to INH (MIC of >1 μg/ml) and shows both (M- and T-catalase activities.

Comparison of molecular differences and similarities between catalases-peroxidases of the main pathogenic mycobacteria such as *M. tuberculosis* and the opportunistic *M. fortuitum* could contribute to our understanding of the molecular mechanisms involved in the death or survival of bacteria within the host. In addition, the implication of mycobacterial T-catalases other than those of *M. tuberculosis* in bacterial susceptibility to INH might give insight to our understanding about the reasons for the resistance of other mycobacteria to the drug.

In this paper, we report the identification and study of two different *katG* genes (*katGI* and *katGII*) encoding proteins with T-catalase activities in *M. fortuitum*. Both genes were also expressed using *E. coli* and *M. aurum* hosts.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. Mycobacteria were grown in Löwenstein-Jensen medium (Difco) and further subcultured in Dubos medium containing Tween-albumin enrichment (Difco) or heart infusion broth (HIB; Difco) and 20 μg of kanamycin per ml when required. *E. coli* strains were grown on LB medium, supplemented when necessary with ampicillin (100 μg/ml) or kanamycin (40 μg/ml).

Catalase activity gels. Protein extracts for nondenaturing polyacrylamide gel electrophoresis (ND-PAGE) (10% [wt/vol] polyacrylamide gel) were carried out as described previously (34, 42). Catalase and peroxidase activities of mycobacterial extracts were examined by the double-staining method as described previously (37). Heat-labile and heat-stable enzymes were distinguished by heat treatment of extracts at 68°C for 1 min before ND-PAGE.

Recombinant DNA techniques and hybridizations. Genomic DNA isolation, restriction enzymes digestion, Southern blotting, and hybridizations were performed as described elsewhere (12). A 700-bp internal fragment of the *katG* gene of *M. intracellulare* was obtained by PCR and used as a probe. For DNA amplification, we used the following oligonucleotides: CAT1 (5′-TTC ATC CGC ATG GCC TGG CAC GGC GCG GGC ACC TAG CGC-3′) (43) and CAT4.1 (5′-GCT GTT GTC CCA CTT CGT-3′; positions 1063 to 1080 [positions correspond to those on the *M. intracellulare katG* gene [24]). Amplification mixes (50 μl) contained 100 ng of genomic mycobacterial DNA plus 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 to 2.5 mM $MgCl_2$, 1 μM each primer, 400 μM each deoxynucleoside triphosphate, and 1.5 U of *Taq* polymerase (Perkin-Elmer). Reactions were subjected to 30 cycles of 60 s at 94°C, 30 s at 58°C, and 1 min at 72°C, with subsequent 5-min extensions. Probe was labeled by using the Prime-α-Gene system (Promega) and 20 μCi of [α-^{32}P]dCTP (Amersham) according to the manufacturer's protocol.

E. coli and *M. aurum* were transformed by electroporation, and colony blots were prepared by standard methods (34). The same conditions for incubation and washes were used in Southern and colony blot hybridization experiments.

DNA sequence and computer analysis. Plasmid sequences were determined by using both universal pUC primers (Pharmacia) and synthetic oligonucleotides (Isogen) and Sequenase version 2.0 as specified by the manufacturer protocol (U.S. Biochemical) with [α-^{35}S]dATP or [α-^{33}P]dATP (Amersham and ITISA, respectively). Both strands were sequenced independently. Amino acid and nucleotide sequences were determined and aligned to closely related ones from data banks (GenBank and SwissProt), using the Genetics Computer Group package program (version 8).

INH resistance test. Tests for inhibition of mycobacterial growth by INH were performed as described previously (8), with some modifications. Briefly, saturated cultures (5 days) of *M. aurum* (wild type and transformants) and *M. fortuitum* on HIB were diluted (1/10) with fresh medium. A drop (5 μl) of diluted culture was spread on plates with medium supplemented with serial dilutions of

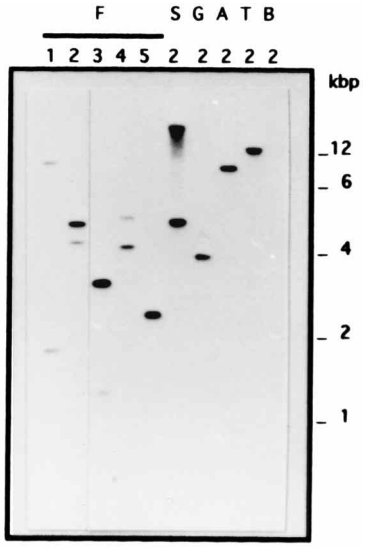

FIG. 1. Southern blot hybridization of restriction enzymes digested mycobacterial DNAs against *M. intracellulare katG* probe (see Materials and Methods). Lanes: F, *M. fortuitum*; S, *M. smegmatis*; G, *M. gadium*; A, *M. avium*; T, *M. tuberculosis* 79500; B, *M. tuberculosis* B1453. Restriction enzymes: lane 1, *Bam*HI; lane 2, *Eco*RI; lane 3, *Pst*I; lane 4, *Sac*I; lane 5, *Sal*I.

INH (0.025 to 10 μg/ml). Three milliliters of HIB was inoculated with 30 μl of saturated cultures; HIB liquid media were also supplemented with increasing amounts of INH (0.025 to 10 μg/ml). The transformants were grown in the presence of kanamycin (20 μg/ml) to assess the maintenance of the plasmids. Three different *M. aurum* transformants were studied for each plasmid. *M. aurum* cultures were incubated at 30°C, and *M. fortuitum* cultures were incubated at 37°C.

Nucleotide sequences accession numbers. The sequences reported here have been deposited in the EMBL data bank with the following accession numbers: Y07865 (*katGI* gene) and Y07866 (*katGII* gene).

RESULTS

Cloning of the *katG* genes of *M. fortuitum*. To detect the *katG* gene in *M. fortuitum*, we used an internal fragment from the *M. intracellulare katG* gene as a probe (see Materials and Methods). Southern blotting of *M. fortuitum* ATCC 6841 genomic DNAs digested with *Bam*HI, *Eco*RI, *Pst*I, and *Sac*I were hybridized with this probe. The presence of two bands with those enzymes indicated a possible internal site for each restriction enzyme (Fig. 1). A 4.3-kbp *Eco*RI fragment and a 4.5-kbp *Sac*I fragment were selected for cloning.

Partial libraries of *M. fortuitum* genomic DNA were prepared by eluting appropriately sized fragments of *Eco*RI- and *Sac*I-digested DNAs from agarose gels and cloning them into pUC18. Two clones, containing each of the corresponding fragments (pMFR [*Eco*RI 4.3-kbp insert] and pMFP [*Sac*I 4.5-kbp insert]), were then identified by colony blot hybridization. Further analysis showed that each clone was coding for a different *katG* gene.

Restriction maps and partial nucleotide sequences of the inserts from pMFR and pMFP were determined. An open reading frame (ORF) was detected in each insert (Fig. 2A); their coding regions correspond to sequences with 2,256 (pMFR) and 2,199 (pMFP) nucleotides. Comparison of the putative amino acid sequences of both ORFs with entries in the GenBank and EMBL databases suggested that these ORFs could correspond to two *katG* genes. They were named *katGI* (pMFR) and *katGII* (pMFP) (Fig. 2A).

Putative ribosome-binding sites were identified 5 bp upstream from the ATG codon (AAAGGGA in pMFR and GA AAGGA in pMFP). In addition, palindromic sequences that had strong sequence similarities to the rho-independent terminators in *E. coli* (31) were also found downstream from *M. fortuitum katGI* and *katGII* genes. The region downstream from the *katGII* gene was closely related to similar sequences also present downstream from the *katG* genes in *M. tuberculosis* (16) and *M. intracellulare* (24) (data not shown).

Search in the data banks detected sequences downstream from the *katGI* gene that showed strong homology with a previously described region, localized in *M. fortuitum* FC1, which flanks the Tn*610* transposon. This region had been cloned into plasmid pIPC17 by Martín et al. (19). Further analysis of pIPC17 by restriction enzyme digestion, hybridization, and sequencing determined the presence of a copy of the *katGI* gene in its insert (Fig. 2). Figure 2A shows the location and orientation of *katGI* in plasmid pIPC17 (corresponding to *M. fortuitum* FC1) and in plasmid pMFR (corresponding to *M. fortuitum* ATCC 6841). Tn*610* is not present in *M. fortuitum* ATCC 6841 (18a).

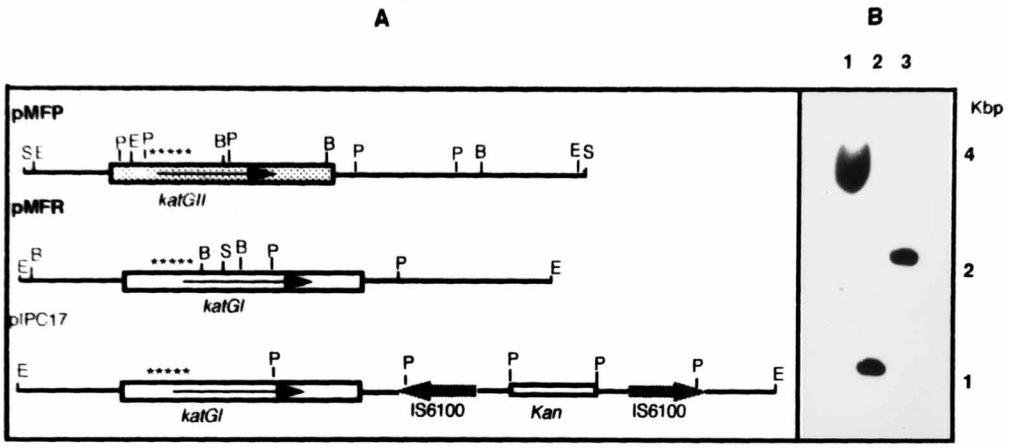

FIG. 2. *M. fortuitum* T-catalase genes. (A) Restriction maps of plasmids pMFR, pMFP, and pIPC17, showing locations and orientations of T-catalase genes. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sac*I. The locations of insertion sequence IS*6100* and the kanamycin-resistant cassette in pIPC17 are also indicated. The position of nucleotide sequences homologous to the catalase probe is indicated by *****. (B) Southern blot hybridization of *Pst*I-digested plasmid DNAs against an *M. intracellulare katG* probe. Lanes: 1, pMFR; 2, pMFP; 3, pIPC17.

A

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                1                                                          60
KatGI M.fort.   MPPNTPDASD ARPPQADTET HSHSESENPV IESPKPKAHA PLTNQDWWPD QVDVSRLHKQ
KatGII M.fort.       MAEAE TH--IGESQ- EPA.E-GC-M ..RI--PVEG G.S-R----N A-NLKI-Q-N

     61                                                                   130
I .-  PIEGNPLGAG FNYAEEFQKL DVEALRADML ELMTSSQDWW PRDYGTYAGL FIRMSWHAAG TYRIFDGRGG
II.-  -PAID-SDE- YD.S-AVKS- ----FQR-FD --LTN----- -A-F-H-GP- -V-------- ---V------

    131                                                                  200
I .-  AGQGAQRFAP INSWPDNVSL DKARRLLWPI KQKYGNKISW ADLIIFAGN. VALESAGFKT FGFAFGRQDI
II.-  G-R-M-P--- L--------- ---------L -K---KQ--- S---VYS--R .-M-HM---- A------P--

    201                                                                  270
I .-  WEPEE.ILWG QEDTWLGTDK RYGGTNDSTN RELANPYGAT TMGLIYVNPE GPEGKPDPLA AAHDIRETFG
II.-  -----D-Y-- A-AE---SQD --A-A-GDRT K.-E--PXXP H--------- ----N--Y-- --I-------

    271                                                                  340
I .-  RMAMNDEETA ALIVGGHTIG KTHGPGPGDL VGPEPEAAPI EQQGLGWKCA FGSGKGSDTI TSGLEVVWTT
II.-  ------I--- --------H- ----ATDIE. N-V---XX-L --M----A.. .NP-L-N--V S-----T--Q

    341                                                                  410
I .-  TPTKWSNSYL EILYGYEWEL TKSPGDAWQF EAKD..AEAI IPDPFGGPPR KPTMLVTDIS MRVDPIYGPI
II.-  H----D--F- ----SN---- ----AG-N-W KP--NGWANS W-MAQ-TGKT H-S--T--L- --F-----E-

    411                                                                  480
I .-  TRRWLEHPEE LNEAFAKAWY KLLHRDMGPI SRYLGPWIP. EPQLWQDPVP DVDHPLVDEQ DIAALKEKLL
II.-  -----D---- -A-EY----F --I------V T-----LV-K QTW----II- AGKQ..LSDA -V-T--AAIA

    481                                                                  550
I .-  DSGLSVQQLV KTAWSAAASF RGTDKRGGAN GGRLRLQPQR NWEVNEP.SE LDKALPVLER IAQDFNASAS
II.-  -----I---- N---K----Y -SS-M---.- ---------L G------E.- -APVIAK--E -Q......-A

    551                                                                  620
I .-  DGKKISLADL IVLGGSAAIE KAARDGGYEV KVHFVAGRTD ASQENTDVDS FAVLEPRADG FRNFVRPGDK
II.-  SDSGV-F--- V----VVGL- --IKAA-FD- A-P-TS-PR- -L--Q----- --Y---KG-- -----AK--S

    621                                                                  690
I .-  APLEQLLVDK AYFLNLTAPE MTVLVGGLRA LNTNHGGSKH GVFTANPGAL SNDFFVNLLD MSTEWKPSET
II.-  V-A-YR-I-R -NL-A-SR-Q ----I----V -GA-----EL --L-DKV-Q- T--Y----T- -G-K-A-APA

    691                                                                  759
I .-  AENVYEGRDR RTGQTRWTAT ANDLVFGSNS VLRAVAEVYA QEDNKAKMIE DFVAAWVKVM NNDRFDLD.
II.-  DDGT-V-T-- A--SPK---S RV--L----- Q---L----- ED-S-E-FVK ------T--- -A-----EA
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B

	M. fortuitum KatGI	*M. fortuitum* KatGII
M. intracellulare	79%	60%
M. bovis	63%	64%
M. tuberculosis	59%	62%
E. coli	60%	51%
S. typhimurium	58%	51%
B. stearothermophilus	43%	40%
R. capsulatus	45%	42%

FIG. 3. Amino acid sequences of *M. fortuitum* T-catalases. (A) Alignment of amino acid sequence of T-catalases from *M. fortuitum*. I, KatGI; II, KatGII. Peroxidase motifs are boxed; identical amino acid positions are indicated by dashes; deletions that maximize alignment are denoted by periods; X's denote ambiguities. (B) Percentages of identity in comparisons of amino acid sequences of T-catalases from *M. fortuitum* (KatGI and KatGII) to T-catalases from other bacterial origins.

Analysis of predicted amino acid sequences of *M. fortuitum* T-catalases. *M. fortuitum* T-catalases (KatGI [752 amino acids] and KatGII [733 amino acids]) showed predicted protein molecular masses of 83 and 80 kDa, respectively. Their amino acid sequences had 79% homology, with 21% conservative changes and 55% identity (Fig. 3A).

Active-site motifs (cataloged from PROSITE 1997) typical of peroxidases were found upon screening of *M. fortuitum* the T-catalase primary structure (Fig. 3A). We also identified either Leu or Ala replacing the more frequent Arg-463 found in the *M. tuberculosis* wild-type catalase-peroxidase (17) (position 480 in Fig. 3A), and the position corresponding to Thr-275 in *M. tuberculosis* wild-type catalase-peroxidase is conserved in both catalases-peroxidases of *M. fortuitum* (position 292 in Fig. 3A).

Comparison of the deduced amino acid sequences for KatGI and KatGII with those described for catalases-peroxidases from nonmycobacterial organisms revealed a degree of identity lower than 60% at the amino acid level (Fig. 3B). However, the level of amino acid identity was greater than 59% when *M. fortuitum* T-catalases were compared with other mycobacterial T-catalases (Fig. 3B). KatG protein of *M. intracellulare* showed the highest value compared with KatGI (79%).

Expression of the *M. fortuitum* T-catalases. To study whether the transcriptional and translational signals of *katG* genes were provided by sequences from the cloned DNA fragments, two different bacterial hosts were selected: *E. coli* DH5α and *M. aurum* L1 (Table 1). *E. coli* was transformed with pMFR and pMFP separately by electroporation, and ampicillin-resistant colonies were selected. Inserts from pMFP (4.5 kbp) and pMFR (4.3 kbp) (Fig. 2A) were cloned into the *Sca*I site of the mycobacterial shuttle vector pRR3 (30). The new plasmids, designated pRMFR and pRMFP, respectively, were electroporated into *M. aurum* L1, and kanamycin-resistant colonies were selected. Protein extracts were prepared from all previously described transformants and tested for peroxidase activity (Fig. 4A and C). Activity bands corresponding to cloned DNA were clearly observed in the cell extracts of *E. coli* and *M. aurum* transformants but absent in wild-types *E. coli* and *M. aurum*. Heat-labile characteristic of the enzymes was demonstrated by ND-PAGE of protein extracts after heat treatment at 68°C for 1 min (Fig. 4A).

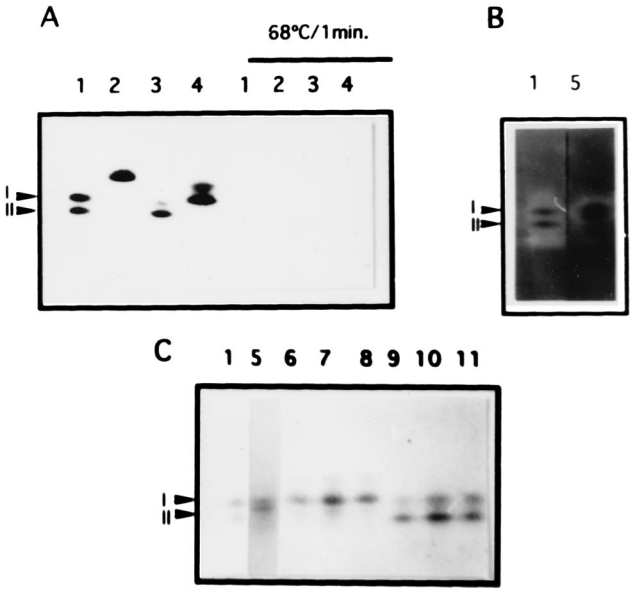

FIG. 4. Expression of *M. fortuitum* T-catalases in other bacteria. Protein extracts from *E. coli* and *M. aurum*, wild type and transformants, as well as *M. fortuitum* were subjected to ND-PAGE and stained for enzymatic activity. (A and C) peroxidase activity; (B) double staining for catalase and peroxidase activities. Catalase activity appears as a white band, and peroxidase activity appears as a dark band. (A) Lanes: 1, *M. fortuitum*; 2, *E. coli* wild type; 3, *E. coli* transformed with pMFP; 4, *E. coli* transformed with pMFR. Thermolability was checked by heat treatment of protein extracts at 68°C for 1 min before electrophoresis. (B) Comparison of catalase-peroxidase activity bands migration between *M. fortuitum* and *M. aurum*. Lanes: 1, *M. fortuitum*; 2, *M. aurum* wild type. (C) Lanes: 1, *M. fortuitum*; 5, *M. aurum* wild type; 6 to 8, *M. aurum* transformed with pRMFR; 9 to 11, *M. aurum* transformed with pRMFP.

The presence of a recombinant plasmid (pRMFR or pRMFP) in transformed mycobacteria was checked by genomic DNA hybridization with the catalase probe (not shown).

ND-PAGE analysis of peroxidase activity in several *M. fortuitum* strains revealed the presence of two identical bands of heat-labile enzyme activity in all strains checked (Fig. 5).

Influence of *katG* genes of *M. fortuitum* on INH resistance. *M. aurum* is a fast-growing mycobacterial species which has been described as being similar to *M. tuberculosis* with respect of INH susceptibility. We therefore chose *M. aurum* as a suitable host to check whether the presence of actively expressed T-catalases of *M. fortuitum* was related to the acquisition of INH resistance.

M. aurum (wild type and transformed with pRR3, pRMFR, and pRMFP) and *M. fortuitum* were grown with increasing

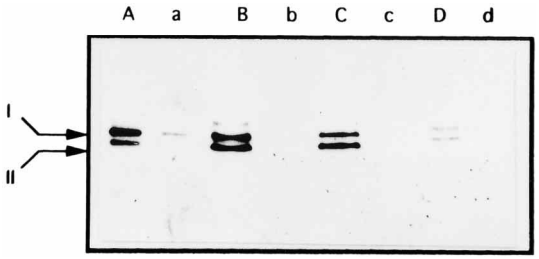

FIG. 5. *M. fortuitum* nondenaturing polyacrylamide gels stained for peroxidase activity. Protein extracts for the following *M. fortuitum* strains were used: lanes A and a, ATCC6841^T; lanes B and b, FC1; lanes C and c, clinical isolate 1250; lanes D and d, ATCC 13756. Lanes corresponding to protein extracts after heat treatment at 68°C for 1 min are indicated by lowercase.

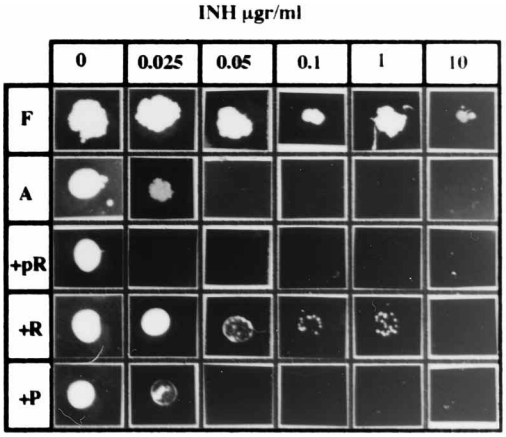

FIG. 6. INH susceptibility of *M. aurum* transformants, determined by colony growth on solid medium after 9 days of incubation of *M. fortuitum* as well as *M. aurum*, both wild type and transformed with several plasmids. Plates were supplemented with increasing amounts of INH (see Materials and Methods). F, *M. fortuitum*; A, *M. aurum*; +pR, *M. aurum* transformed with plasmid pRR3; +R, *M. aurum* transformed with plasmid pRMFR; +P, *M. aurum* transformed with plasmid pRMFP.

amounts of INH (see Materials and Methods). Figure 6 shows that all bacteria tested were inhibited by INH at 10 μg/ml. In addition, wild-type *M. aurum* and *M. aurum* transformed with plasmid pRR3 were also inhibited with as little as 0.05 μg of the drug per ml. However *M. aurum* transformed with the plasmid carrying *katGI* (pRMFR) and *M. fortuitum* grew in the presence of up to 1 μg of INH per ml. Finally, growth of *M. aurum* transformed with pRMFP (carrying the *katGII* gene) was inhibited by drug concentrations greater than 0.05 μg/ml (Fig. 6). Results obtained when susceptibility tests were performed on liquid media were comparable to those described previously for tests performed on solid plates. Thus, *M. fortuitum* growth was inhibited by 10 μg of INH per ml, and growth of *M. aurum*, both wild type and transformed with the plasmid pRR3, was inhibited by 0.1 μg/ml; finally, *M. aurum* transformed with a plasmid carrying each of the *katG* genes from *M. fortuitum* was inhibited by 0.5 μg/ml as for pRMFP and 5 μg/ml as for pRMFR. Differences in the MIC for tests on solid or liquid media have been often described.

DISCUSSION

In this study, we have shown that *M. fortuitum* has two dissimilar genes (*katGI* and *katGII*) encoding two enzymes (KatGI and KatGII) with the same catalase-peroxidase activity. Multicopy genes in mycobacteria such as the rRNA operons in fast growers (4, 9) genes encoding heat shock proteins or fibronectin binding protein antigens in slow growers (41) have previously been shown to be highly homologous. This is the first report of two dissimilar genes encoding same enzyme function which are actively expressed within the same mycobacterium. Genes encoded within repetitive elements, such as insertion sequences, have been previously described as multiple genes encoding transposition (20, 21). However, their enzymatic activity has been only indirectly demonstrated in mycobacteria.

M. fortuitum katG genes are 64% homologous at the nucleotide level, and their corresponding amino acids sequences have only 55% identity (Fig. 3A), which is similar to the range observed when they are compared with catalases-peroxidases from different bacteria (Fig. 3B). It is possible that the origin of the duplication observed was due to gene duplication and

subsequent evolutionary divergence in a progenitor of *M. fortuitum* or due to horizontal transmission from another bacterium into a more recent *M. fortuitum* strain. The finding in one of the *M. fortuitum* strains studied, FC1, of sequences corresponding to transposon Tn*610* in the vicinity of the *katGI* gene (Fig. 2) might suggests the latter as likely.

Changes inside coding and/or regulatory gene sequences have been related to mobile genetic elements. We have detected a copy of Tn*610* neighboring the *katGI* gene in *M. fortuitum* FC1 with no apparent changes in gene product (Fig. 2 and 5). A similar result was described previously for IS*6110* and the *sodA* gene in *M. tuberculosis* (44). These two examples show that insertion sequences can also produce silent mutations when inserted into bacterial genomes.

Previous results obtained by our group suggested the possibility of more than one T-catalase actively expressed in other fast-growing mycobacteria such as *M. smegmatis* (10).

E. coli and the fast grower *M. aurum* were transformed with genes coding for T-catalases of *M. fortuitum*. Expression of the two enzymes was detected in both cases (Fig. 4A and C), probably indicating that both genes use their own regulatory sequences in both hosts. Expression of mycobacterial genes in *E. coli* is difficult, probably due to differences in the −35 regions of mycobacterial promoters (3). The results for expression in *E. coli* of *M. tuberculosis* and *M. fortuitum* T-catalases (reference 43 and this report) as well as *M. avium* M-catalase (23) show a more general regulatory mechanism in the expression of catalases than has been described for other mycobacterial genes involved in the oxidative clearance such as *sodA* (22, 42).

One of the main characteristics described for mycobacterial T-catalases is their relationship with INH susceptibility in *M. tuberculosis*. INH resistance in some strains is due to a loss of catalase-peroxidase activity caused by complete deletion of the *katG* gene or by point mutations as detected in the majority of resistant *M. tuberculosis* strains (17, 32). The functional importance of these mutations has not yet been elucidated, but they could confer advantages for adaptation to a particular host niche and/or variation in substrate interaction by substrate-binding-site modification (17, 25, 33). *M. fortuitum* is considered INH resistant, possibly because KatGI and KatGII are unable to activate INH efficiently.

M. aurum has a level of INH sensitivity similar to that of *M. tuberculosis* and has therefore been used to study the molecular mechanisms of INH resistance in mycobacteria (8, 39). We have used *M. aurum* as a host to check the influence of each *M. fortuitum* T-catalase in acquisition of INH resistance. We found that *katGI*-transformed *M. aurum* acquired a level of INH resistance similar to that of *M. fortuitum*, whereas *katGII*-transformed mycobacteria showed a level of resistance similar to that of wild-type *M. aurum* (Fig. 6 and Results). These results suggest that each T-catalase of *M. fortuitum* may have a different affinity and therefore activation efficiency for INH. It is noteworthy that KatGII is more similar to T-catalase of INH-sensitive *M. tuberculosis* than KatGI (Fig. 3B). These results are in agreement with description of more than one point mutation as responsible for catalase-dependent INH resistance in mycobacteria and also indicate possible dissimilar contributions of different T-catalases to INH resistance in mycobacteria other than *M. tuberculosis.*

More studies are needed to elucidate the complex mechanisms involving mycobacterial catalases. An understanding of these differences and similarities when one is comparing pathogenic and nonpathogenic mycobacteria could provide novel insights into the mechanisms of INH resistance and survival within macrophages.

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