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The catalase–peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*

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TUBERCULOSIS is responsible for one in four of all avoidable adult deaths in developing countries[1]. Increased frequency and accelerated fatality of the disease among individuals infected with human immunodeficiency virus has raised worldwide concern that control programmes may be inadequate[2], and the emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* has resulted in several recent fatal outbreaks in the United States[3]. Isonicotinic acid hydrazide (isoniazid, INH) forms the core of antituberculosis regimens; however, clinical isolates that are resistant to INH show reduced catalase activity and a relative lack of virulence in guinea-pigs[4–7]. Here we use mycobacterial genetics[8,9] to study the molecular basis of INH resistance. A single *M. tuberculosis* gene, *katG*, encoding both catalase and peroxidase, restored sensitivity to INH in a resistant mutant of *Mycobacterium smegmatis*, and conferred INH susceptibility in some strains of *Escherichia coli*. Deletion of *katG* from the chromosome was associated with INH resistance in two patient isolates of *M. tuberculosis*.

Most strains of *M. tuberculosis* are highly susceptible to INH (minimum inhibitory concentration, $IC_{min} < 0.02\ \mu g\ ml^{-1}$). Other species of mycobacteria show a range of sensitivities to INH, saprophytic mycobacteria such as *M. smegmatis*, for example, being susceptible to only high concentrations (IC_{min}, $32\ \mu g\ ml^{-1}$) (Fig. 1). In order to identify the *M. tuberculosis* genes involved in INH resistance, a mutant strain of *M. smegmatis*, BH1, which is able to grow at $500\ \mu g\ ml^{-1}$ INH, was isolated[10] and transformed with a set of shuttle cosmid clones containing inserts of about 30 kilobases (kb) with a representative coverage of genomic DNA from *M. tuberculosis* H37Rv. Transformants were tested for growth on plates containing $32\ \mu g\ ml^{-1}$ INH and one of them, pBH4, was found to confer hypersensitivity to INH (IC_{min} $8\ \mu g\ ml^{-1}$) (Fig. 1). Minimum inhibitory concentrations for other antituberculosis drugs were unchanged. BH1 has reduced catalase activity by comparison with the parent strain, MC^2155 (refs 9, 10), but introduction of pBH4 led to 2–3-fold overproduction of this enzyme (Fig. 1).

Analysis of the catalase activity in *M. tuberculosis* indicated that it resembled the hydroperoxidase I (KatG) enzyme of *E. coli* in being heat-labile and having an associated peroxidase activity[11]. As a strategy for cloning the relevant gene from *M. tuberculosis*, a series of oligonucleotide probes was designed on the basis of amino-acid sequences conserved between hydroperoxidase I enzymes of *E. coli* and *Bacillus stearothermophilus*[12,13]. In Southern blot experiments one of these probes, corresponding to amino-acid residues 99–111 of the *E. coli* enzyme identified a 4.5-kb fragment generated by *Kpn*I digestion of genomic DNA from *M. tuberculosis* H37Rv. A partial library was prepared from appropriately sized fragments of *Kpn*I-digested DNA in the vector pUC19 and a clone containing the corresponding fragment, pYZ55, was isolated by colony hybridization. Transformation of *E. coli* with pYZ55 resulted in expression of peroxidase and catalase activities with electrophoretic mobilities identical to those in extracts of *M. tuberculosis*, and nucleotide sequence analysis identified an open reading frame encoding a protein with marked homology to *E. coli* hydroperoxidase I (Fig. 2).

Restriction enzyme mapping and Southern blot hybridization revealed that pBH4, the cosmid responsible for restoration of INH susceptibility, and pYZ55 contained the same 4.5-kb fragment (Fig. 1). Subsequent transformation of BH1 with this fragment in a mycobacterial shuttle plasmid was shown to confer INH sensitivity; this activity was encoded by a 2.9-kb *Eco*RV–*Kpn*I subfragment with coding capacity for the catalase–peroxidase alone (Fig. 1). Inactivation of *katG* by various 3′ deletions resulted in loss of both enzyme activity and ability to confer INH susceptibility (Figs 1 and 2).

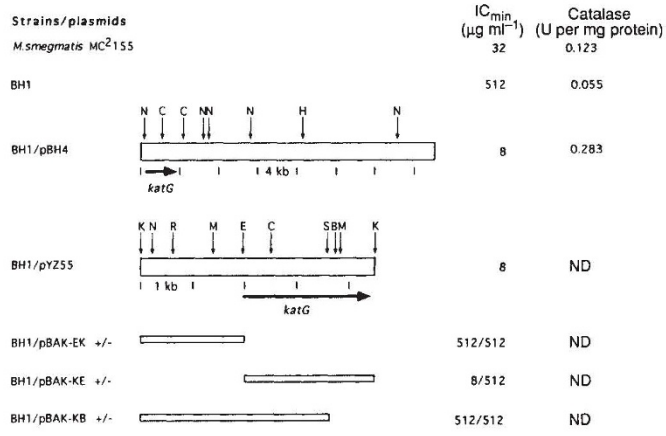

FIG. 1 Restriction map of the DNA insert from pBH4 is shown with that of the insert from pYZ55—a plasmid containing *katG* of *M. tuberculosis* H37Rv, isolated on the basis of hybridization with an oligonucleotide probe (5′-TTCATCCGCATGGCCTGGCACGGCGCGGGCACCTACCGC-3′) designed to match the amino-acid sequence from a conserved region of *E. coli* hydroperoxidase I. Restriction sites for the following enzymes are indicated: B, *Bam*HI; C, *Cla*I; E, *Eco*RV; H, *Hind*III; K, *Kpn*I; M, *Sma*I; N, *Not*I; R, *Eco*RI; S, *Sac*I. The INH-resistant *M. smegmatis* strain, BH1 (ref. 10); a derivative of strain MC^2155 (ref. 9) was transformed with a pool of *M. tuberculosis* H37Rv shuttle cosmids (provided by W. R. Jacobs, New York) and individual clones were scored for INH susceptibility. Cosmid pBH4 consistently conferred drug susceptibility and the transformant overproduced catalase (assayed as described[10]). Transformation of BH1 with a mycobacterial shuttle plasmid, pBAK14 (ref. 18), which contained the 4.5-kb insert from pYZ55, likewise conferred INH susceptibility. IC_{min} values are also shown for BH1 transformed with subfragments derived from pYZ55 and inserted into pBAK14 in one (+) or other (−) orientation. The *katG* gene and the ability to confer INH susceptibility both mapped to a 2.9-kb *Eco*RV–*Kpn*I fragment (pBAK − KE+). ND, not determined.

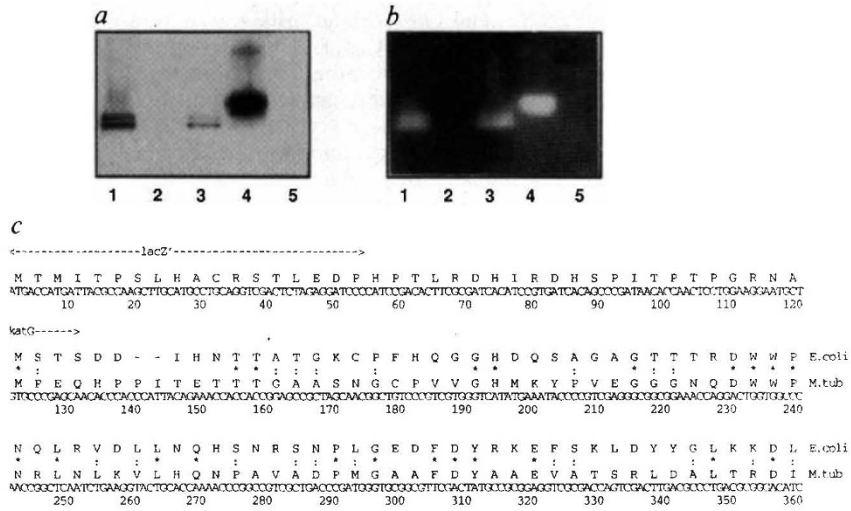

FIG. 2 Extracts from *M. tuberculosis* H37Rv and from *E. coli* strains transformed with a variety of plasmid constructs were prepared for activity gel analysis as described[18]. Non-denaturing gels containing 8% polyacrylamide were stained for *a*, catalase and *b*, peroxidase activities as described[19]. Lane 1, *M. tuberculosis* H37Rv; lane 2, *E. coli* UM2 (*katE, katG*; ref. 14); lane 3, *E. coli* UM2/pYZ55; lane 4, *E. coli* UM2/pYZ56 (the 2.9-kb *Eco*RV–*Kpn*I fragment in pUC19, corresponding to pBAK-KE+ in Fig. 1); lane 5, *E. coli* UM2/pYZ57 (pYZ55 with a BamHI–*Kpn*I deletion, corresponding to pBAK−KB+ in Fig. 1). *M. tuberculosis* catalase and peroxidase activities migrated as two bands under these conditions (lane 1); the same pattern was seen for the recombinant enzyme expressed by pYZ55 (lane 3). pYZ56 (lane 4) expresses a protein of increased M_r owing to a fusion between *katG* and lacZ′ from the vector shown in *c*. *c*, Partial sequence alignment with *E. coli* hydroperoxidase I (the complete sequence of the gene will be communicated elsewhere).

Escherichia coli is completely insensitive to INH. To assess the possibility that expression of the *M. tuberculosis katG* gene could confer INH sensitivity in *E. coli*, various strains were transformed with constructs expressing different levels of the enzyme (Fig. 2). No effect was seen in wild-type *E. coli*, but overexpression of the *M. tuberculosis* gene in two catalase-negative strains (UM2 and UM255, both *katG, katE*; ref. 14) resulted in susceptibility to high INH concentrations (Fig. 3). Expression of the enzyme at a lower level was less effective in conferring drug susceptibility (Fig. 3).

Many INH-resistant isolates of *M. tuberculosis* have decreased catalase activity, with the most highly resistant isolates (IC_{min} > 50 μg ml^{-1}) being completely catalase-negative[4–7]. To examine this phenomenon at the genetic level, chromosomal DNA from a panel of INH-sensitive and INH-resistant strains of *M. tuberculosis* was probed by Southern hybridization using the 4.5-kb *Kpn*I fragment containing the catalase–peroxidase gene (Fig. 4). In two highly resistant isolates (strain B1453, and strain 24; Fig. 4*a*, lanes 3 and 4) the catalase probe produced no signal at all, whereas in the remaining strains (including a low-resistance isolate, strain 12; IC_{min}, 1.6 μg ml^{-1}), a 4.5-kb *Kpn*I fragment identical to that in strain H37Rv was observed. Reprobing of the same blot with the superoxide dismutase gene gave a strong signal with both of the INH-resistant isolates (Fig. 4*b*), showing that the lack of hybridization with the catalase probe was not due to insufficient DNA. We have previously described an additional genetic change in strain B1453 which results in a restriction fragment length polymorphism like that seen with the superoxide dismutase gene probe (Fig. 4*b*, lane 3)[15]. From a panel of eight INH-resistant isolates, with IC_{min} of 1.6 to >50 μg ml^{-1}, *katG* deletion was observed in two out of three highly-resistant strains (IC_{min} > 50 μg ml^{-1}). We conclude that, in a subset of INH-resistant patient isolates, the loss of catalase activity is due to deletion of the catalase gene.

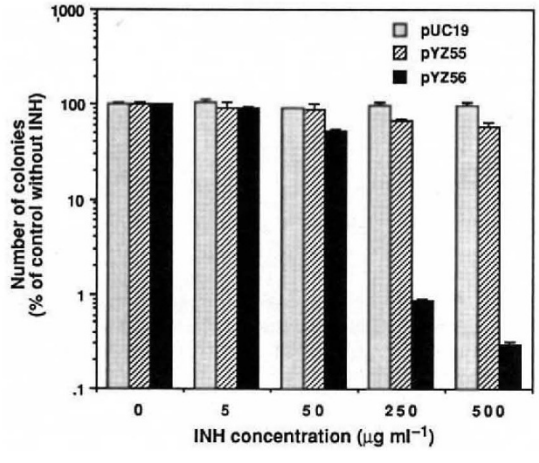

FIG. 3 An *E. coli* strain with mutations in both *katG* and *katE* (UM2; ref. 14) was transformed with pUC19 vector alone (stippled bars), pYZ55 expressing *M. tuberculosis katG* (hatched bars) and pYZ56 with high-level expression of *M. tuberculosis katG* (solid bars). Overnight cultures in Luria–Bertani broth supplemented with appropriate antibiotics were plated out in the presence of varying concentrations of INH and colony-forming units were assessed. Results of a representative experiment are shown with error bars indicating the standard deviation in triplicate samples. Overexpression of *M. tuberculosis katG* similarly conferred susceptibility to high concentrations of INH in *E. coli* UM255 (*katG, katE*; ref. 14), but had no effect on catalase-positive strains such as *E. coli* TG1.

Mycolic acid biosynthesis, NAD^+ and pyridoxal phosphate metabolism have all been proposed as possible targets for isoniazid[6,7,16]. Our evidence demonstrates a key role for the catalase–peroxidase enzyme in the action of INH. Deletion of the catalase–peroxidase gene in INH-resistant isolates of *M. tuberculosis* may be an event that is coincident with the deletion of an adjacent undefined gene, but the fact that the catalase–peroxidase gene alone can confer sensitivity to INH in the *M. smegmatis* mutant and in catalase-negative *E. coli* shows that this enzyme must be important in the action of INH. We anticipate that transformation of INH-resistant *M. tuberculosis* isolates with the catalase–peroxidase gene will also restore sensitivity to INH, but transformation experiments with these strains using standard shuttle vectors have so far been unsuccessful. It has been proposed that the catalase–peroxidase enzyme may be important in converting INH to a metabolically active form in the cell[7,11], or in the INH-dependent generation of reactive oxygen radicals[17]. Availability of the cloned *katG* gene will facilitate the testing of such hypotheses.

Gene deletion represents an unexpected and unusual mechanism for development of drug resistance. Effective drugs must be active against cell components that are essential for bacterial viability, and resistance is generally conferred either by an altered structure of the drug target, or by acquisition of an efficient drug degradation system or permeability barrier. It is likely that in *M. tuberculosis* other forms of INH resistance may also occur. Inactivation of the catalase–peroxidase gene by movement of an insertion element, or point mutations, are attractive theoretical possibilities, and screening of extended panels of INH-resistant isolates of *M. tuberculosis* will be required to assess the relative frequency of gene deletion compared with other potential mechanisms of INH resistance. The

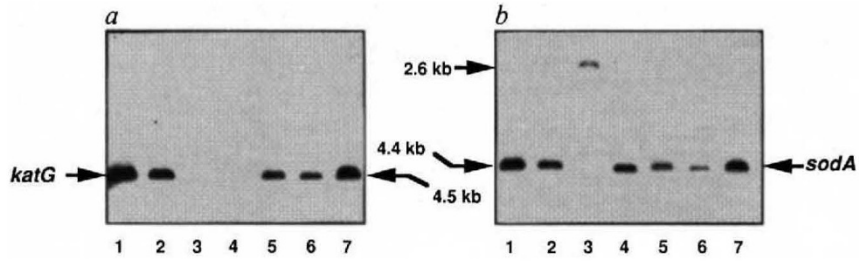

FIG. 4 Southern blots prepared using genomic DNA from different *M. tuberculosis* strains, digested with *KpnI*, were probed with *a*, *katG* (the 4.5-kb *KpnI* fragment), and *b*, the *sodA* gene (1.1-kb *EcoRI–KpnI* fragment; ref. 18). Labelling of probes and processing of blots was as described[15]. Lane 1, H37Rv; lane 2, strain 12, IC_{min} 1.6 μg ml^{-1} INH; lane 3, B1453, $IC_{min} > 50$ μg ml^{-1} INH[20]; lane 4, strain 24, $IC_{min} > 50$ μg ml^{-1} INH; lane 5, 79112, INH-sensitive[21]; lane 6, I2646, INH-sensitive[21]; lane 7, 79665, INH-sensitive[21]. INH susceptibilities were confirmed by inoculation of Lowenstein-Jensen slopes containing differing concentrations of INH.

multiple-drug-resistant strains in which there is a correlation between INH resistance and decreased catalase activity are particularly important because, owing to the contagiousness of tuberculosis, these strains pose a public health threat to both HIV-infected and healthy individuals[3]. An improved understanding of the mechanisms of drug resistance will enable rapid tests for drug-resistance isolates to be developed and should facilitate the design of antituberculosis drugs. □

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Anatomy of a transcription factor important for the Start of the cell cycle in *Saccharomyces cerevisiae*

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ENTRY of yeast cells into the mitotic cell cycle (Start) involves a form of the CDC28 kinase that associates with G1-specific cyclins encoded by *CLN1* and *CLN2* (ref. 1). The onset of Start may be triggered by the activation of *CLN1* and *CLN2* transcription in late G1 (ref. 2). SWI4 and SWI6 are components of a factor (SBF) that binds the CACGAAAA (SCB) promoter elements[3–5] responsible for activation in late G1 of the *HO* endonuclease, *CLN1* and *CLN2* genes[6,7]. A related factor (MBF) containing SWI6 and a 120K protein[8] binds to the ACGCGTNA (MCB) promoter elements responsible for late G1-specific transcription of DNA replication genes[9–12]. Nothing is known about how these heteromeric proteins bind DNA. We show here that SWI4 contains a novel DNA-binding domain at its N terminus that alone binds specifically to SCBs and a C-terminal domain that binds to SWI6. SWI4's DNA-binding domain is similar to an N-terminal domain of the *cdc10* protein that is a component of an MBF-like factor from *Schizosaccharomyces pombe*[13] and is required for Start[14,15]. An involvement of this kind of DNA-binding domain in transcriptional controls at Start may therefore be a conserved feature of eukaryotic cells.

To determine whether SWI4 or SWI6 alone can bind SCBs, we translated both proteins in reticulocyte lysates. Full-length SWI6 (846 residues[16]) is made efficiently but much of SWI4 (1,094 residues[4,17]) is either degraded or prematurely terminated (Fig. 1*a*). We tested the ability of the proteins to bind an oligonucleotide from the *CLN2* promoter (pCL2) that contains three potential SCBs and forms a complex with SBF isolated from yeast[6]. Using a gel retardation assay, we observe a heterogenous set of SWI4:pCL2 complexes (Fig. 1*b*), all of which are recognized by a SWI4-specific antibody but not by preimmune serum (data not shown and Fig. 1*c*). The heterogeneity may be due to the variable size of the SWI4 protein. No complexes were observed using the SWI6 protein (Fig. 1*b*). That SWI4 but not SWI6 can bind SCB DNA is consistent with experiments showing that SWI4 overproduction allows *HO* to be transcribed without SWI6[17,18] (but not vice versa) and that *CLN2* can be partially activated by SWI4 in *swi6* mutants[6,19].

Cotranslated SWI4 and SWI6 proteins form a new complex (with pCL2) containing both proteins that migrates with a mobility similar to that of the complexes formed by partially purified SBF from yeast (Fig. 1*b*, *c*). The complex formed with *in vitro* translated proteins seems to migrate slightly faster than that formed by yeast proteins and could conceivably lack a third component or modification. A truncated version of SWI4 lacking 144 amino acids from its C-terminal end (SWI4E) cannot form complexes with SWI6 although it still binds pCL2 (Fig. 1*d*). *HO* expression due to modest overproduction of such a protein in yeast is largely SWI6-independent[17]. Likewise, a version of SWI6 lacking its most C-terminal 89 amino acids (SWI6C) cannot form complexes on pCL2 with SWI4 (Fig. 1*e*). SWI4 and SWI6 might therefore interact by their C termini. This part of SWI6 is highly conserved in *Kluyveromyces lactis*