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Regulation of catalase–peroxidase (KatG) expression, isoniazid sensitivity and virulence by *furA* of *Mycobacterium tuberculosis*

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Summary

Mycobacterium tuberculosis **has two genes for ferric uptake regulator orthologues, one of which, *furA*, is situated immediately upstream of *katG* encoding catalase–peroxidase, a major virulence factor that also activates the prodrug isoniazid. This association suggested that *furA* might regulate *katG* and other genes involved in pathogenesis. Transcript mapping showed *katG* to be expressed from a strong promoter, with consensus −10 and −35 elements, preceding *furA*. No promoter activity was demonstrated downstream of the *furA* start codon, using different gene reporter systems, indicating that *furA* and *katG* are co-transcribed from a common regulatory region. The respective roles of these two genes in the isoniazid susceptibility and virulence of *M. tuberculosis* were assessed by combinatorial complementation of a Δ(*furA–katG*) strain that is heavily attenuated in a mouse model of tuberculosis. In the absence of *furA*, *katG* was upregulated, cells became hypersensitive to isoniazid, and full virulence was restored, indicating that *furA* regulates the transcription of both genes. When *furA* alone was introduced into the Δ(*furA–katG*) mutant, survival in mouse lungs was moderately increased, suggesting that FurA could regulate genes, other than *katG*, that are involved in pathogenesis. These do not include the oxidative stress genes *ahpC* and *sodA*, or those for siderophore production.**

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Introduction

The current well-documented tuberculosis pandemic has refocused research efforts on the biology and physiology of *Mycobacterium tuberculosis*, with the specific goals of developing a vaccine with greater efficacy than the currently available BCG and novel antituberculosis compounds. These goals are only likely to be achieved by elucidating the molecular basis of pathogenicity and virulence. The development of genetic tools for manipulating the slow-growing mycobacterium *M. tuberculosis* (Pelicic *et al*., 1998), in conjunction with the complete genome sequence of *M. tuberculosis* H37Rv (Cole *et al*., 1998), has already resulted in the identification of a growing number of genes required for optimal survival within macrophages and animal models (Collins *et al*., 1995; Berthet *et al*., 1998; Camacho *et al*., 1999; Buchmeier *et al*., 2000; De Voss *et al*., 2000; Glickman *et al*., 2000; McKinney *et al*., 2000). However, little is known about the regulation of these and other virulence factors. Analysis of the *M. tuberculosis* genome revealed the existence of over 100 putative transcriptional regulators (Cole *et al*., 1998), undoubtedly reflecting the varied microenvironments that this organism encounters within the host, which require adaptive alterations in gene expression.

One of the first determinants of virulence to be identified was the catalase–peroxidase. After the initial observation that isoniazid-resistant clinical isolates of *M. tuberculosis* were frequently catalase negative and of lower virulence in guinea pigs (Middlebrook and Cohn, 1953; Middlebrook, 1954), it was subsequently demonstrated that the *katG* gene encodes the sole catalase–peroxidase of *M. tuberculosis* (Zhang *et al*., 1992), and mutations within or deletions of this gene result in attenuation of virulence and isoniazid (INH) resistance (Heym *et al*., 1995; Wilson *et al*., 1995; Rouse *et al*., 1996; Li *et al*., 1998). Biochemical studies of recombinant and mutant KatG have also shown that this protein is required for the activation of INH, thus accounting for its role in conferring INH resistance (Johnsson *et al*., 1997; Saint Joanis *et al*., 1999). It has been proposed that KatG protects *M. tuberculosis* against the reactive oxygen intermediates encountered within the activated macrophage, as catalase and peroxidase activities are related to survival within cultured macrophages (Manca *et al*., 1999).

Although KatG has been well established as a virulence factor, little is known about its regulation. *M. tuberculosis* lacks a functional *oxyR* gene (Deretic *et al*., 1995; Sherman *et al*., 1995), which regulates KatG and other proteins induced by exposure to H_2O_2 in *Escherichia coli*. However, the complete genome sequence of *M. tuberculosis* revealed that there are two *fur* gene orthologues, one of which, *furA*, is situated immediately upstream of *katG*, with the two genes seemingly constituting an operon. The transcription factor *fur* (ferric uptake regulation) in *E. coli* is principally involved in the regulation of genes required for iron acquisition (Escolar *et al*., 1999). In contrast, *M. tuberculosis* possesses a gene, *ideR*, that has been shown in *Mycobacterium smegmatis* to be functionally equivalent to *E. coli fur* (Dussurget *et al*., 1996). This suggested that the *fur* genes of *M. tuberculosis* might be transcriptional regulators of genes involved in functions other than the acquisition of iron (De Voss *et al*., 1999). This is the case in *Bacillus subtilis*, an organism with three Fur orthologues, one of which, PerR, has been shown to regulate genes involved in the oxidative stress response, including the catalase KatE (Bsat *et al*., 1998). Given the genomic association of *furA* and *katG*, we hypothesized that FurA might be involved in the regulation of *katG*. We were also intrigued by the possibility that *furA* of *M. tuberculosis* might regulate other genes involved in virulence.

In this paper, we present evidence that *furA* and *katG* represent an operon that is autoregulated by *furA* in a negative fashion. In addition, using a Δ(*furA–katG*) deleted strain of *M. tuberculosis* complemented with *furA*, *katG* or both genes, we have evaluated the effects of these individual genes on virulence in a mouse model of tuberculosis.

Results

KatG is co-transcribed with furA

The position of *furA,* situated 40 bp upstream of *katG*, suggested that the two genes might be co-transcribed. As a first step in defining the transcriptional organization of *furA* and *katG*, reverse transcription–polymerase chain reaction (RT–PCR) mapping of the *katG* transcript was carried out. Using three primers, it was possible to amplify, from total RNA extracted from *M. tuberculosis* after a reverse transcription step, a fragment extending from positions +48 to either position −159 or −401 relative to the *katG* start codon. However, it was not possible to amplify a fragment using two additional primers situated at positions −178 and −780, indicating that the 5′ end of the *katG* transcript lay somewhere between positions −401 and −780 relative to the *katG* start codon. Further definition of the 5′ end of this transcript was achieved using S1 nuclease protection analysis. A single-stranded probe, generated with a primer located at position −379, protected a product from S1 nuclease digestion when hybridized with mRNA extracted from *Mycobacterium bovis* BCG. When compared with the sequencing ladder, the 5′ end of this transcript mapped to position −481, which corresponded to the start codon of the *furA* gene (Fig. 1A). The putative −10 and −35 regions for this transcript bore close similarity to the consensus sequences for mycobacterial promoter elements (Fig. 1B) (Gomez and Smith, 2000), confirming that position −481 represented a genuine transcriptional start site and was not simply a product of post-transcriptional modification.

*Characterization and complementation of a Δ(*furA–katG*) strain of* M. tuberculosis

To determine whether *furA* regulated the expression of *katG*, a strategy involving the combinatorial complementation of a Δ(*furA–katG*) strain of *M. tuberculosis* was adopted to avoid the possibility of polar effects that might result from the use of a mutant with insertional inactivation of *furA.* From our collection of INH-resistant clinical isolates of *M. tuberculosis*, a strain, INH34, previously shown by PCR to have a deletion of *katG* (Heym *et al*., 1994; 1995), was selected for further study. No catalase activity or expression was detected in protein extracts from this strain using activity gels and immunoblotting with an anti-KatG polyclonal antibody (data not shown), confirming the absence of *katG.* Southern hybridization of *Eco*RI-digested genomic DNA with a radioactively labelled *furA* PCR probe produced a single appropriately sized band with H37Rv (10 781 bp) genomic DNA but not with DNA from INH34, indicating that a fragment encompassing the *furA–katG* locus of this strain had been deleted during selection for INH resistance (Fig. 2B).

To determine precisely the extent of this deletion, a chromosomal walking strategy was used to identify a pair of primers (located at positions 5′-2152868-87 and 5′-2159257-38 on the *M. tuberculosis* H37Rv chromosome; Cole *et al*., 1998) that could PCR amplify a fragment spanning the *furA–katG* deletion when using genomic DNA from strain INH34. Nucleotide sequencing of this 450 bp fragment revealed that the *katG* deletion was 5925 bp in size, extending from position 2153067 to 2158991 of the H37Rv sequence. This was predicted to result in the complete loss of *katG*, *furA* and the adjacent genes Rv1907c, Rv1910c and *lppC*, as well as the disruption of *fadB5* (Fig. 2A). This deletion was flanked on either side by an 11 bp perfect repeat, suggesting a possible substrate for recombination.

As described in *Experimental procedures*, a series of constructs was assembled to complement strain INH34

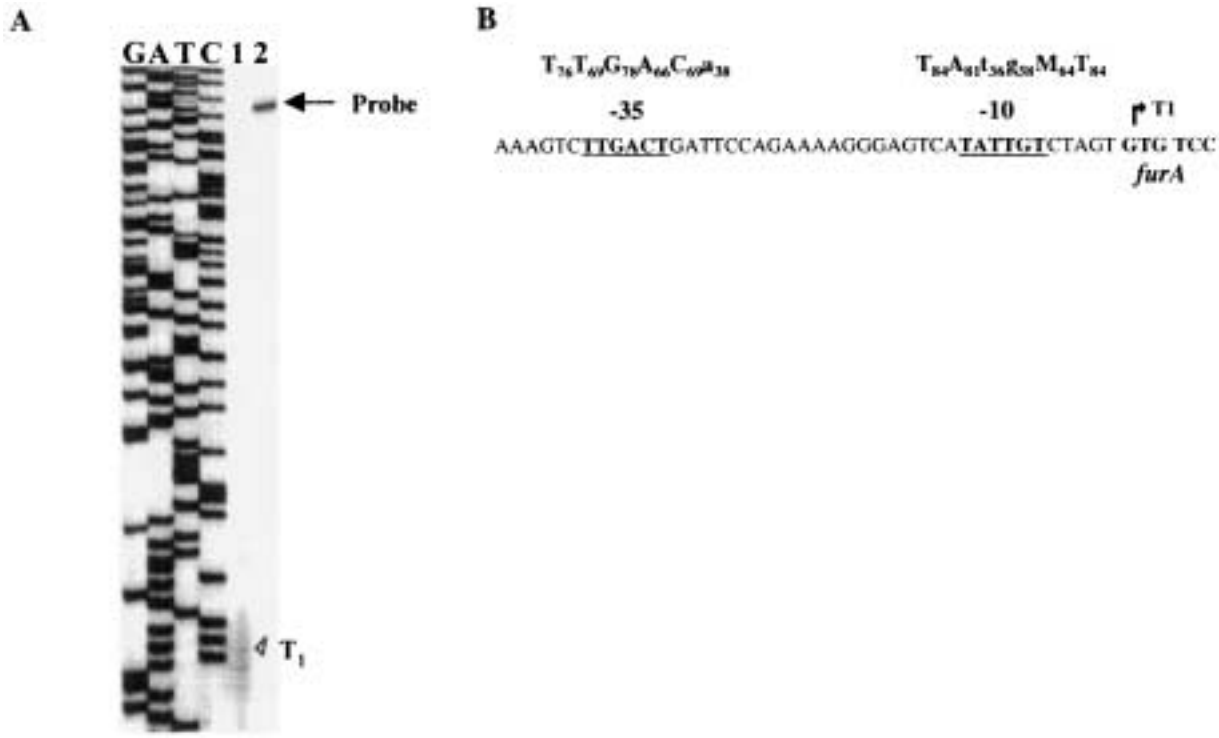

Fig. 1. A. Mapping of the 5′ end of the *katG* transcript by S1 nuclease protection analysis using RNA extracted from *M. bovis* BCG. The protected product (lane 1) and the undigested probe (lane 2) were run alongside a standard sequencing ladder (complementary sequence) generated by the primer and template used to synthesis the probe. T1 corresponds to the 5′ end of the transcript coinciding with the first G of the *furA* start codon.
B. Nucleotide sequence upstream of the 5′ end of the *furA–katG* transcript showing the putative −10 and −35 regions and the consensus sequences for these promoter elements in *M. tuberculosis*. The numbers represent percentage conservation, and M corresponds to a C or an A.

(Fig. 3) with *furA*, *katG* or *furA–katG*. For the two constructs harbouring only the *katG* gene, the region upstream of *katG* in pAP5 was identical to that of pPD28 for 394 bp, corresponding to the 40 bp intergenic region and 354 bp of the 3′ end of *furA* and, for pAP8, it was identical to pPD28 except for a 354 bp deletion of the 3′ end of *furA*.

Full expression of the katG *gene is dependent on the promoter upstream of* furA

To assess the promoter activity of the *furA* and *katG* upstream regions in *Escherichia coli*, the various pKINT-based constructions were transformed into UM262, an *E. coli katG* knock-out mutant, and catalase expression of crude protein extracts was evaluated using activity gel electrophoresis (Fig. 4A). Although both pPD28- and pAP8-transformed UM262 produced high levels of catalase activity, no catalase activity was seen with the pAP5 transformant, indicating that there was no significant promoter activity located in the 394 bp region immediately upstream of *katG*. As only a proportion of mycobacterial promoters are recognized in *E. coli*, the same constructs were transformed into strain INH34 to evaluate whether there was a mycobacterial-specific promoter in this region. Expression of catalase was initially monitored by activity gel electrophoresis using crude protein extracts from late log phase cultures. As with *E. coli*, these revealed that there was no detectable KatG expression in INH34 transformed with pAP5, but high levels with pPD28 and pAP8. To quantify the levels of catalase expression in the crude protein extracts of INH34 transformed with either pPD28 or pAP8, catalase activity was determined spectrophotometrically (Table 1), and catalase expression was determined by immunoblotting with an anti-KatG polyclonal antibody (Fig. 4B). This revealed that there was an approximately fivefold increase in catalase activity in crude protein extracts from INH34/pAP8 compared with those from INH34/pPD28 and H37Rv, and that this relative increase in catalase activity was also detectable by immunoblot analysis. In contrast, there was no activity seen in the extracts from INH34 transformed with pAP5, indicating that the region immediately upstream of *katG* had little or no demonstrable promoter activity in *M. tuberculosis*. These changes in catalase activity were also reflected in the sensitivity to INH, as INH34/pAP8 was 5–10 times more sensitive to INH than either H37Rv or INH34 transformed with pPD28 (Table 1).

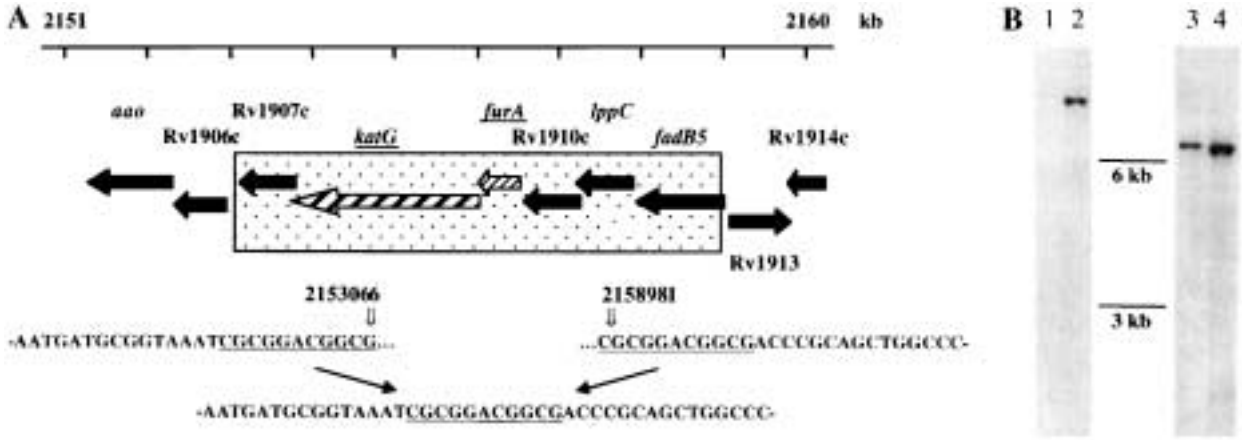

Fig. 2. A. Map of the deleted region in *M. tuberculosis* strain INH34. Arrows represent the open reading frames annotated in the H37Rv genome sequence, with ascribed gene names above or below (http://genolist.pasteur.fr/TubercuList/). The hatched area corresponds to the deleted fragment, with the end sequences and H37Rv genome co-ordinates given below. The 11 bp identical repeat is underlined.
B. Southern blot analysis of *Eco*RI-digested genomic DNA from strains INH34 (lanes 1 and 3) and H37Rv (lanes 2 and 4). The membrane was first blotted with a probe corresponding to the *furA* gene (lanes 1 and 2) and subsequently rehybridized with a probe encompassing the *kasA* gene (lanes 3 and 4) to demonstrate that digested DNA from both strains had been transferred adequately.

To quantify the promoter activity of the region upstream of *katG*, appropriate PCR fragments amplified from H37Rv genomic DNA were cloned in front of the promoterless *luxAB* gene incorporated in a multicopy mycobacterial shuttle vector pSG10 (Gordon *et al*., 1998). After transformation of *M. smegmatis*, the promoter activity of these various constructs was determined by light emission (Fig. 5). The construct bearing the region directly upstream of *katG* (pAP13) was found to have 11-fold less activity than the region directly upstream of *furA* (pAP12), whereas the construct containing the complete *furA–katG* region (pAP9) had 2.5-fold less activity than the region upstream of *furA*. Although *M. smegmatis* also possesses a *furA* gene that could conceivably interact with the pSG10 constructs, these results confirm that *katG* expression is dependent on the promoter upstream of *furA* and that the presence of *furA* results in diminished expression of *katG*.

FurA *of* M. tuberculosis *does not regulate* ahpC, sodA *or siderophore production*

The *fur* genes of *E. coli* and a variety of other organisms (Escolar *et al*., 1999) regulate genes required for iron acquisition. To determine whether *furA* of *M. tuberculosis* also regulates siderophore production, strain INH34 and the transformant of INH34 complemented with *furA* were grown in a minimal medium with or without ferric chloride, and the supernatants were assayed for siderophores. Both INH34 transformed with the control plasmid and INH34 transformed with the plasmid-bearing *furA* showed repressed siderophore production when the strains were grown in iron-rich conditions, indicating that the absence of *furA* did not alter the expression of siderophores in these strains (data not shown). No discernible difference in halo size was seen when these strains were grown on CAS indicator plates.

Like *M. tuberculosis*, *B. subtilis* possesses more than one *fur* gene. These have recently been characterized, and one of them has been shown to regulate genes

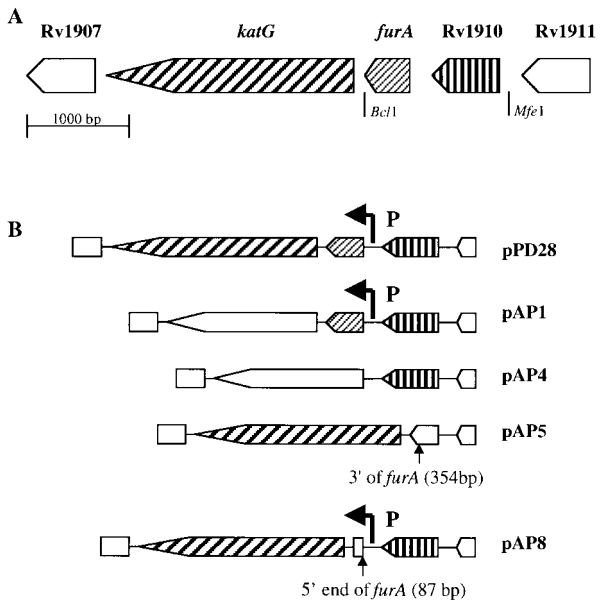

Fig. 3. A. Diagram of the *furA–katG* locus of *M. tuberculosis* showing the principal restriction sites used for cloning.
B. Maps of the fragments cloned into the *Eco*RI site of the mycobacterial integration vector pKINT. Shaded genes represent complete genes, and unshaded genes are interrupted or partial. The promoter upstream of *furA* is represented by **P**, when present within the fragment.

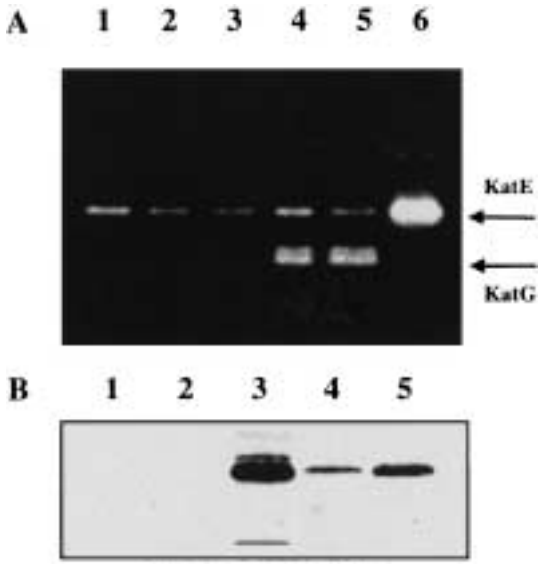

Fig. 4. A. Catalase activity gel of protein extracts from *E. coli* UM262 transformed with various *M. tuberculosis katG*-bearing plasmids. The upper band represents KatE of *E. coli,* and the lower double band represents KatG from *M. tuberculosis*. A total of 5 μg of protein was run in each well of a non-denaturing 8% polyacrylamide gel. Lanes 1–5, UM262 transformed with pAP4, pAP1, pAP5, pPD28 and pAP8, respectively; and lane 6, *E. coli* DH5α.
B. Immunoblot analysis with anti-KatG antibody (*M. tuberculosis*) of protein extracts from *M. tuberculosis* strain INH34 transformed with the same constructs. Lanes 1–4 are proteins from INH34 transformed with pAP4, pAP5, pAP8 and pPD28, respectively, and lane 5 from *M. tuberculosis* H37Rv.

involved in protection against oxidative stress (Bsat *et al*., 1998). To determine whether *furA* of *M. tuberculosis* might regulate genes other than KatG that protect against oxidative stress, the expression of AhpC and SodA was examined in the *furA* deleted and complemented strain (Fig. 6). Whole-cell protein fractions were prepared from mid-log phase cultures and subjected to immunoblotting with either polyclonal anti-AhpC or anti-SodA antibody. No difference was seen between the levels of expression of SodA in the *furA* deleted and complemented strains and reference strains. Similarly, there was no demonstrable effect of *furA* on the expression of AhpC, although as expected, the INH34-derived strains showed upregulation of AhpC when compared with H37Rv and the INH-resistant strain INH26 (used as an additional control), as a result of a previously characterized point mutation in the *ahpC* promoter region (Heym *et al*., 1997).

Regulation of virulence factors by furA

To investigate whether *furA*-regulated genes, other than *sodA*, *ahpC* or the siderophores of *M. tuberculosis*, might be involved with virulence, the transformants of INH34 complemented with *furA*, *katG* or *furA–katG* were assayed with an intravenous infection model using BALB/C mice. After inoculation with 5×10^6 colony-forming units (cfus), the growth of each strain was monitored in the spleen and lung (Fig. 7). As anticipated from previous studies (Wilson *et al*., 1995; Li *et al*., 1998), the transformants harbouring a *katG* gene were more virulent than the control strain, as both grew more vigorously in the spleens and lungs 25 days after infection. INH34 transformed with *katG* alone appeared to be more virulent than the transformant harbouring the *furA–katG* construct, suggesting that overexpression of catalase–peroxidase can increase virulence. When the transformant harbouring *furA* alone was compared with the control strain, there was no statistical difference between cfus for each strain in the spleen but, after 40 days, the cfus of the *furA* transformant in the lungs were significantly more numerous than those of the control. This suggests that *furA* probably regulates genes, other than *katG*, that are involved in the survival of *M. tuberculosis* within mouse lungs.

FurA *gene polymorphisms and isoniazid resistance*

Having established the transcriptional organization of the *furA and katG* genes, a panel of INH-resistant clinical isolates of *M. tuberculosis* was analysed to determine whether mutations in the promoter elements of this operon, which could lead to decreased expression of KatG and therefore decreased activation of INH, were associated with INH resistance. Using two pairs of primers, the region extending from the *katG* initiation codon to 300 bp upstream of *furA* was amplified by PCR from 36 INH-resistant isolates of *M. tuberculosis* and subjected to automated sequencing. No mutations relative to the H37Rv genome sequence were detected in the region upstream of *furA* but, in two strains, a point mutation (a G to C and a G to A at positions 205 and 291 of *furA*) was detected within the *furA* coding region, giving rise to the amino acid substitutions Leu-68Phe and

Table 1. Specific catalase activity and sensitivity to isoniazid of *M. tuberculosis* strain INH34 transformants.

Strain	Genotype *furA*	Genotype *katG*	Specific catalase activity (UE min^{-1} mg^{-1})	MIC[a] for INH (μg ml^{-1})
INH34/pAP4	–	–	ND[b]	> 10
INH34/pAP1	+	–	ND	> 10
INH34/pAP5	–	+	ND	> 10
INH34/pAP8	–	+	0.154 ± 0.018[c]	0.01
INH34/pPD28	+	+	0.039 ± 0.011	0.1
H37Rv	+	+	0.081 ± 0.025	0.05–0.1

a. Minimal inhibitory concentration.
b. Not detected.
c. Mean and standard deviation of five experiments.

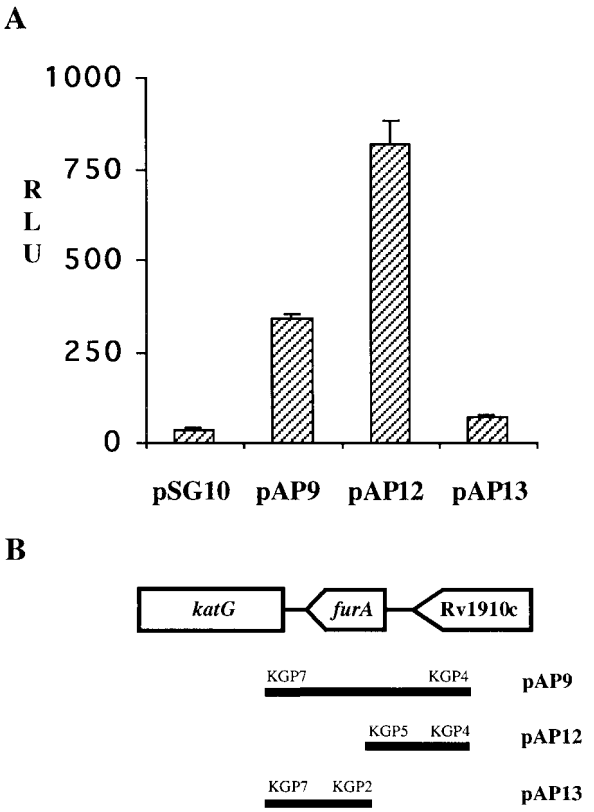

Fig. 5. A. Promoter activity in *M. smegmatis* of constructs harbouring different portions of the region upstream of *katG*, cloned in front of the promoterless reporter gene *luxAB*. Light emissions are expressed in relative light units (RLUs) per 1 μl of a culture with an optical density of 1 at 600 nM. Results are the mean of three experiments, and error bars correspond to the standard deviation.
B. Positions relative to *furA* of the PCR-generated fragments directionally cloned into the *luxAB* reporter gene vector pSG10. Four primers, KGP2, KGP4, KGP5 and KGP7, with an internal *Bam*HI or *Xba*I were used, and their 5′ H37Rv genome co-ordinates are 2156522, 2156899, 2156487 and 2156063 respectively.

Cys-97Tyr. Although both these strains also had mutations in *katG* (Ser-315Thr and f-Met-1Ala respectively), which probably account for their elevated minimum inhibitory concentration (MIC) for INH (5 μg ml^{-1}), it is possible that, by altering the DNA-binding properties of FurA, these mutations could lead to additional changes in KatG expression that might affect the sensitivity to INH.

The same regions were also amplified and sequenced from two strains of *M. bovis* (AF2122/97 and the MDR strain MB7) and from five vaccine strains of *M. bovis* BCG (Russia, Sweden, Prague, Connaught and Pasteur 1173P2). Two point mutations were detected relative to the H37Rv sequence, one (a C to A at position −286 relative to the start codon of *furA*) was detected in all the strains tested; the other (a C to T at position 128 of the *furA* gene leading to Ala-43Asp substitution) was found exclusively in the BCG strains. A mutation present in all these vaccine strains, including BCG Russia (presumed to be closest to the virulent progenitor strain; Behr *et al.*, 1999), raises the possibility that it arose during and contributed to the attenuation process of BCG, although Ala-43 does not appear to be a highly conserved residue.

Discussion

In this paper, we have commenced the characterization of the regulatory role of the *furA* gene of *M. tuberculosis*. We have shown that the 5′ end of the *katG* transcript maps to position −481 relative to its start codon, corresponding to the start codon of the *furA* gene, and is associated with appropriately positioned −10 and −35 promoter elements. This suggested that *furA* and *katG* could be co-transcribed from a common regulatory region upstream of *furA*. This was confirmed when the various regions upstream of *katG* were assayed for promoter activity using two separate sets of constructs, with either *luxAB* or *katG* itself as a reporter gene. Whereas no significant promoter activity could be demonstrated downstream of the *furA* start codon, strong activity was detected in the region immediately upstream of *furA*. However, we only evaluated the promoter activity *in vitro*, and it is possible that, during infection or in response to specific stimuli, *katG* could be transcribed independently of *furA*. This is currently being investigated. We also observed that the inclusion of an intact *furA in cis* with the region upstream of *furA* reduced promoter activity when compared with the region upstream of *furA* alone. In

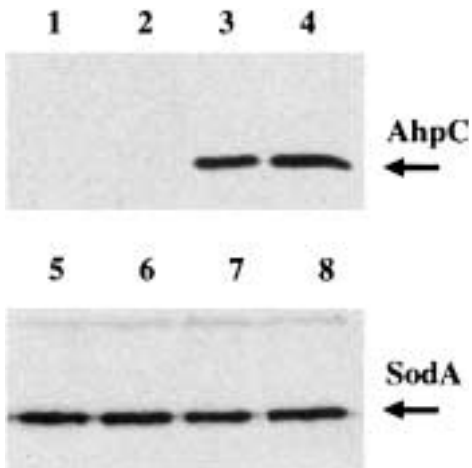

Fig. 6. Immunoblot analysis of crude protein extracts using either an anti-AhpC antibody (lanes 1–4) or anti-SodA (lanes 5–8) and 25 μg of protein from H37Rv (lanes 1 and 5), *M. tuberculosis* INH26 (lanes 2 and 6) and *M. tuberculosis* INH34 transformed with either pAP4 (lanes 3 and 7) or pAP1 lanes (4 and 8). Strain INH26 is an isoniazid-resistant strain without an *ahpC* promoter mutation, which is included as an additional control.

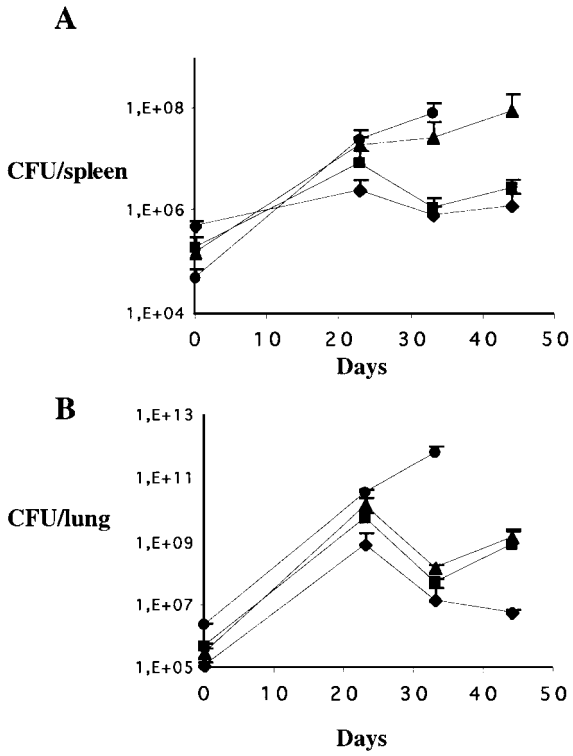

Fig. 7. Growth of transformants of INH34 in (A) mouse spleens or (B) mouse lungs (● INH34/pAP8; ■ INH34/pAP1; ▲ INH34/pPD28; ◆ INH34/pAP4) after intravenous injection with 5×10^6 bacteria. Error bars are the standard deviation of the mean of three or four mice per time point. Seven of the INH34 pAP8 cohort of mice died after the fifth week after infection, and one each from the pAP1 and pPD28 cohorts during the seventh week after infection.

addition, the construct (pAP8) in which the 3′ end of *furA* had been deleted also overexpressed KatG. Taken together, this indicates that *furA* inhibits its own transcription and therefore that of *katG* as well. This genomic organization of a *fur* gene upstream of a catalase is conserved across a wide range of mycobacteria (Pagan-Ramos *et al*., 1998) and streptomyces. In both *Streptomyces coelicolor* and *Streptomyces reticuli*, studies have demonstrated that these Fur proteins regulate the respective catalase–peroxidases in the same manner as that proposed for *M. tuberculosis* (Zou *et al*., 1999; Hahn *et al*., 2000; Ortiz de Orue Lucana and Schrempf, 2000). The strong sequence homology between the identified DNA-binding motif for the *S. reticuli* Fur protein and the H37Rv genome sequence at an equivalent position upstream of *furA* indicates the probable site of fixation of FurA in *M. tuberculosis*.

INH is a prodrug that requires activation by KatG before it can exert its antimycobacterial effect (Zhang *et al*., 1993; Johnsson *et al*., 1997). Therefore, an understanding of the regulation of KatG expression has a number of implications with regard to the INH susceptibility of *M. tuberculosis*. We have shown that overexpression of KatG leads to INH hypersensitivity. This indicates that, at least *in vitro*, the antimycobacterial activity of this drug is limited by the rate of its activation and not by the nature of its interaction with other cellular processes, such as the inhibition of mycolic acid synthesis. The design of INH analogues that are more effectively activated by KatG would therefore be a rational strategy for producing a drug with increased antimycobacterial potency.

INH resistance in clinical isolates of *M. tuberculosis* is most commonly the result of mutations within the *katG* gene, resulting in diminished peroxidation of INH to its active form (Heym *et al*., 1994; Musser *et al*., 1996). Having established that the expression of *katG* is driven by promoter elements situated upstream of the *furA* gene, we were also interested to see whether mutations in this region that would lead to an alteration in KatG expression could be involved in mediating INH resistance. Promoter region mutations of two other genes associated with INH resistance, *inhA* and *aphC* (Heym *et al*., 1994; Musser *et al*., 1996; Sreevatsan *et al*., 1997), have been described in INH-resistant isolates. Although sequencing upstream of the *furA* gene did not reveal any point mutations, two non-synonymous mutations were detected within the *furA* coding region. One of these, cysteine to tyrosine at position 97, is of particular interest, as it is part of the highly conserved motif CysXYCysGly. Substitution of the equivalent cysteine by a serine in *E. coli* results in altered metal-binding properties (Coy *et al*., 1994), suggesting that this mutation would lead to functional changes in FurA. Further biochemical analysis of these mutant proteins is now required to establish what role they may play in the adaptive response to INH or to the loss of catalalase–peroxidase activity associated with mutations in the *katG* gene.

The construction of a panel of isogenic strains with or without *furA* and *katG* also enabled us to begin an analysis of the *furA* regulon in *M. tuberculosis*. We were unable to demonstrate any differences between the *furA* deleted and complemented strain in the repression of siderophore biosynthesis in iron-rich conditions. Although mutations could have arisen during the selection of strain INH34 to compensate for an imbalance in siderophore regulation resulting from the loss of *furA*, our results would suggest that FurA is not the principal regulator of siderophore production. This is compatible with the presence of the gene *ideR* in *M. tuberculosis*, which has been shown to act as a repressor of exochelin and mycobactin synthesis in *M. smegmatis* (Dussurget *et al*., 1996), although the *ideR* mutant of *M. smegmatis* did not have complete derepression of siderophore synthesis in iron-rich conditions, suggesting the presence of a second iron-dependent repressor in mycobacteria. FurB, which is more closely related to *E. coli* Fur than FurA, or the gene Rv2788, which has similarity to an iron-dependent

regulator SirR from *Staphylococcus epidermis* (Hill *et al*., 1998), are two possible candidates for this second repressor (De Voss *et al*., 1999).

We were also unable to demonstrate a regulatory role for FurA in the control of SodA or AhpC expression, suggesting that FurA is not functionally equivalent to the PerR (peroxide regulon repressor) proteins of *B. subtilis* (Bsat *et al*., 1998) and *Campylobacter jejuni* (van Vliet *et al*., 1999), which regulate the expression of AhpC as well as catalase. However, when the different strains were assayed in a mouse model of virulence, INH34 transformed with *furA* was found to be more virulent than INH34 transformed with the vector control, suggesting that *furA* regulates genes other than *katG* that are required for optimal growth in mouse lungs. This is compatible with the observation that complete deletion of *furA* and *katG* is an INH resistance-conferring mutation rarely encountered *in vivo*, suggesting that loss of both *furA* and *katG* results in a more attenuated phenotype than the loss of *katG* only and is less likely therefore to emerge *in vivo*. Although FurA negatively regulates *katG* expression, the mechanism by which FurA controls the expression of other virulence genes can only be speculated. Conceivably, it could act directly as an activator as well as a repressor, or indirectly through interaction with another regulatory pathway.

The conventional approach of systematically characterizing the phenotype of single gene knock-outs, selected from the 4000 genes identified in the *M. tuberculosis* genome (Cole *et al*., 1998), is a highly laborious approach for discovering virulence determinants, given the slow growth and difficulties associated with manipulating this class III pathogen. The identification of a regulator that is implicated in the control of other genes associated with the pathogenesis of *M. tuberculosis* presents an alternative strategy for selecting genes for detailed analysis. Further studies are now required to describe the genes regulated by *furA* that have the potential to identify novel virulence factors of *M. tuberculosis*.

Experimental procedures

DNA preparation, digestion and hybridization

Mycobacterium tuberculosis H37Rv and the INH-resistant strain 34 (*ΔfurA–katG*) were grown in Middlebrook 7H9 medium (Difco) supplemented with albumin–dextrose (ADC; Difco) for 10–12 days. After overnight incubation with glycine (0.2 M final concentration), cells were harvested by low-speed centrifugation and resuspended in lysozyme solution (500 μg ml^{-1} lysozyme, 25% sucrose, 50 mM Tris, pH 8.0, 50 mM EDTA). After incubation for 4 h at 37°C, an equal volume of Proteinase K solution (100 mM Tris, pH 8.0, 400 μg ml^{-1} Proteinase K, 1% SDS) was added and reincubated for a further 4 h at 55°C, before phenol–chloroform extraction and ethanol precipitation to obtain chromosomal DNA. Chromosomal DNA was digested overnight with *Eco*RI (Gibco BRL), migrated through 1% agarose gels and transferred by capillary transfer to a Hybond-C Extra nitrocellulose membrane (Amersham). Probes were labelled with [α-^{32}P]-dCTP (Amersham) using the Prime-It II kit (Stratagene), and unincorporated nucleotides were removed using a P10 minicolumn (Bio-Rad). Hybridizations were carried out overnight at 37°C in 5× SSC (0.75 M NaCl, 0.75 M sodium citrate), 50% (v/v) formamide and 10 mg ml^{-1} denatured salmon sperm. Membranes were washed at 37°C in 2× SSC−0.1% SDS and 1× SSC−0.1% SDS for 15 min each and exposed to X-ray film (Amersham) at −80°C.

PCR and sequencing reactions

Reactions contained 0.25 units of *Taq* polymerase (Gibco), 2.5 μl of 10× PCR buffer (Gibco), 2.5 μl of 20 mM nucleotide mix, 2.5 μl of each primer (2 μM), 5 ng of template DNA, 10% dimethyl sulphoxide (DMSO) and sterile distilled water to 25 μl. Thermal cycling was performed on a PTC-100 amplifier (MJ) with an initial denaturation step of 90 s at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 58°C and 2 min at 72°C. Sequencing reactions were performed using the *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems). Reactions were loaded onto 6% polyacrylamide gels, and electrophoresis was performed on an ABI 373A or 377 automatic DNA sequencer (Applied Biosystems). Sequence data were transferred to a Digital Alpha station and analysed using the TED sequence analysis program from the Staden software package.

RT–PCR and S1 nuclease protection assay

RNA was isolated from *M. bovis* BCG by sedimentation through a cushion of 5.7 M CsCl (Curcic *et al*., 1994). A single-stranded ^{32}P-radiolabelled DNA probe was prepared as described previously (Dhandayuthapani *et al*., 1996) using a specific oligonucleotide primer (5′-TGTGGATGCGCATTCACTGCT-3′), located at position −379 relative to the *katG* start codon, and a single-stranded *furA–katG* template generated from a 1724 bp *Sma*I–*Bam*HI fragment digested from pYZ55 (Zhang *et al*., 1992) and subcloned into an M13-based phagemid vector. The probe was annealed to 50 μg of total RNA and subsequently digested with S1 nuclease (Boehringer Mannheim) (Dhandayuthapani *et al*., 1996). Digestion products were ethanol precipitated and analysed on a sequencing gel alongside the undigested probe, and a sequencing ladder was generated from the primer and template described above. RNA for RT–PCR reactions was prepared from a 1-week-old 10 ml culture of *M. tuberculosis* H37Rv. The bacteria were resuspended in 1 ml of TRIzol reagent (Gibco) after harvesting by low-speed centrifugation and broken using 500 μl of 600-μm-diameter beads and a Mickle apparatus. After centrifugation at 12 000 *g* for 10 min, the supernatant was chloroform extracted, ethanol precipitated and resuspended in 60 μl of DEPC-treated water before treatment with DNase 1 (Boehringer Mannheim). The mRNA was then reprecipitated and resuspended in DEPC-

treated water. RT–PCR reactions were carried out in a 25 μl volume using the Access RT–PCR system (Promega) containing ≈100 ng of mRNA, according to the recommended protocol. Control reactions were carried out in parallel without the addition of AMV reverse transcriptase to exclude the possibility of DNA contamination.

Construction of plasmids for complementation of M. tuberculosis *with* furA *and* katG

In order to investigate the function of *furA*, a series of constructs was created in a mycobacterial integrative vector to complement strain INH34 with *furA*, *katG* or *furA–katG* (Fig. 3). Initially, an *Eco*RI 4.3 kb fragment spanning the entire *furA–katG* region was cloned from pYZ55 into pKINT (a gift from Professor Douglas Young, Imperial College School of Medicine, London, UK) to form pPD28. A 752 bp *Nde*I–*Nco*I fragment was removed from the 5′ end of the *katG* gene of pPD28, which was then religated to form pAP1, a construct harbouring only *furA*. A 575 bp *Nhe*1 fragment spanning *furA* was then excised from pAP1 to form pAP4, the control plasmid without *katG* and *furA*. To create a construct with *katG* but without *furA*, a PCR fragment covering the region directly upstream of either *furA* or *katG* was directionally cloned into pYZ55 digested with *Bcl*I and *Mfe*I. The *Bcl*I site between the *furA* and *katG* genes is situated 39 bp upstream of the *katG* start codon. For the region upstream of *katG*, a fragment generated with primers KGPI (5′ end at H37Rv-2156510 and containing an *Eco*RI site) and TBO4 (5′ end at H37Rv-2156050) was digested with *Eco*RI and *Bcl*I and ligated into pYZ55 to create pAP2. The region upstream of *furA* was generated in the same way using primers KGP6 (5′ end at H37Rv-2157453 and containing an *Eco*RI site) and KGP5 (5′ end at H37Rv-2156487 and containing a *Bam*HI site) and was similarly ligated into pYZ55 to form pAP7. The *Eco*RI fragment from pAP2 and pAP7 was then transferred to the integrative vector pKINT to create pAP5 and pAP8 respectively. The integrity of all these clones was verified by automated sequencing.

Enzyme assays, immunoblotting and drug susceptibility testing

Bacilli from 14-day-old 50 ml cultures were concentrated by centrifugation, washed twice with 50 mM sodium phosphate buffer (pH 7.0) and resuspended in 500 μl of the same buffer. Cells were then lysed by shaking in a Mickle apparatus for 10 min with 500 μl of acid-washed 600-μm-diameter glass beads. The supernatant obtained after centrifugation at 12 000 r.p.m. for 30 min was aliquoted and stored at −20°C before protein quantification using a Bio-Rad protein assay, immunoblotting and specific activity testing. Catalase activity was determined spectrophotometrically by measuring the decrease in H_2O_2 concentration at 240 nm ($\varepsilon_{240} = 0.0435\ mM^{-1}\ cm^{-1}$). The reaction mixture (1 ml) contained 50 mM sodium phosphate buffer, pH 7.0, and 10 mM H_2O_2. Catalase activities were also detected in native 10% polyacrylamide gels according to standard protocols (Heym and Cole, 1992). Immunoblotting was carried out after SDS–PAGE and transfer of proteins to a nitrocellulose membrane (Hybond C+ Amersham), followed by incubation with an anti-KatG (diluted 1:2500), anti-AhpC (diluted 1:1000) (Heym *et al.*, 1997) or anti-SodA (diluted 1:1000) polyclonal antibody. Detection of bound immunoglobulin was achieved using an enhanced chemiluminescence–peroxidase system (Amersham). The MIC for INH was determined by inoculating dilutions of a 7-day 7H9-ADC culture into a 48-well cell culture plate containing 1 ml of fresh medium $well^{-1}$. Each well was supplemented with one of eight concentrations of INH ranging from 0 to 10 μg ml^{-1}, and the plates were read after 1 and 2 weeks to ascertain the MIC.

Transformation of M. tuberculosis *and* M. smegmatis

Electrocompetent cells for INH-resistant *M. tuberculosis* strain 34 were generated from 400 ml of a 10-day-old Middlebrook 7H9-ADC culture. Bacilli were harvested by centrifugation at 3000 *g* for 20 min at 16°C, washed with H_2O at room temperature and resuspended in 1–2 ml of room temperature 10% glycerol after recentrifugation. Bacilli (250 μl) and ≈5 μg of purified plasmid were mixed and electroporated using a Bio-Rad gene pulser with settings of 2.5 kV, 25 μF and 1000 Ω. After electroporation, bacilli were resuspended in 2 ml of Middlebrook 7H9-OADC (Difco) and left overnight at 37°C. Transformants were selected by plating out on Middlebrook 7H11 medium (Difco) supplemented with oleic acid–albumin–dextrose–catalase (OADC; Difco) and 25 μg ml^{-1} kanamycin (Sigma). Kanamycin-resistant colonies appearing after 3 weeks were analysed for the presence of integrated vector by PCR using primers specific for the kanamycin resistance cassette. *M. smegmatis* mc^2155 was transformed using a similar protocol. Bacteria grown to an optical density at 600 nm (OD_{600}) of 0.5–0.8 in L broth containing 0.2% Tween 80 and 0.5% glycerol were harvested by low-speed centrifugation, washed with cold 10% glycerol, recentrifuged and resuspended in 1–2 ml of 10% glycerol. Electroporation was carried out in the same way as for *M. tuberculosis*, with electroporated cells being plated out onto L plates containing 25 μg ml^{-1} kanamycin after incubation at 37°C for 4 h. Kanamycin-resistant colonies appearing after 3–4 days were selected for further analysis by PCR.

Luciferase and siderophore assays

Individual colonies of mc^2155 harbouring the individual *luxAB* reporter gene constructs picked from kanamycin plates were grown for 24 h in L broth containing 0.2% Tween 80, 0.5% glycerol and 25 μg ml^{-1} kanamycin. The cultures were subsequently diluted to an OD of 0.25 using fresh medium and incubated for a further hour. To measure the expression of luciferase, 50 μl of 0.1% nonyl aldehyde in 100% ethanol was added to 500 μl of bacterial culture, and the total light emission in relative light units (RLUs), during a 10 s interval, was quantified using a BioOrbit 1254-001 luminometer. To measure exochelin production, bacteria were grown in plastic containers for 1 month in either a minimal medium (containing 10 g l^{-1} asparagine, 10 g l^{-1} KH_2PO_4, 40 ml l^{-1} glycerol), previously deferrated overnight with Chelex 100 resin (Bio-Rad), or the same medium supplemented with 100 μm

of $FeCl_3$. The supernatants were obtained after centrifugation at 6000 r.p.m. for 15 min and were assayed for exochelin using the Chrome Azurol S assay. Siderophore units were calculated according to the formula $(A_r–A_s)/A_r)100$, where A_r equals absorbance at 630 nm of minimal medium with CAS assay solution, and A_s is the absorbance of sample and assay solution. CAS indicator plates were prepared as described previously (Fiss *et al.*, 1994).

Virulence studies

Cultures (50 ml) of individual strains were grown in parallel in Middlebrook 7H9–ADC medium supplemented with 0.05% Tween 80 and 25 $\mu g\ ml^{-1}$ kanamycin for 7–10 days. Bacteria were harvested by low-speed centrifugation and washed once with 50 mM sodium phosphate buffer (pH 7.0) before resuspension in 1–5 ml of the same buffer. The bacteria were then sonicated briefly and allowed to stand for 2 h to allow residual bacterial aggregates to settle. The bacterial suspensions were then aliquoted and frozen for 48 h at −80°C. A single defrosted aliquot was used to quantify the viable counts (cfu) before inoculation. Six-week-old female BALB/cByJIco mice (IFFA Credo) were infected intravenously via the lateral tail vein with 5×10^6 cfu. Organs from the sacrificed mice were homogenized using a Mickle apparatus and 2.5-mm-diameter glass beads. Serial fivefold dilutions in PBS were plated on 7H11 agar, and cfu were ascertained after 2–3 weeks growth at 37°C.

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References

Behr, M.A., Wilson, M.A., Gill, W.P., Salamon, H., Schoolnik, G.K., Rane, S., and Small, P.M. (1999) Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* **284**: 1520–1523.

Berthet, F.X., Lagranderie, M., Gounon, P., Laurent-Winter, C., Ensergueix, D., Chavarot, P., *et al.* (1998) Attenuation of virulence by disruption of the *Mycobacterium tuberculosis erp* gene. *Science* **282**: 759–762.

Bsat, N., Herbig, A., Casillas-Martinez, L., Setlow, P., and Helmann, J.D. (1998) *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. *Mol Microbiol* **29**: 189–198.

Buchmeier, N., Blanc-Potard, A., Ehrt, S., Piddington, D., Riley, L., and Groisman, E.A. (2000) A parallel intraphagosomal survival strategy shared by *Mycobacterium tuberculosis* and *Salmonella enterica*. *Mol Microbiol* **35**: 1375–1382.

Camacho, L.R., Ensergueix, D., Perez, E., Gicquel, B., and Guilhot, C. (1999) Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. *Mol Microbiol* **34**: 257–267.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., *et al.* (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**: 537–544.

Collins, D.M., de Kawakami, R.P., Lisle, G.W., Pascopella, L., Bloom, B.R., and Jacobs, W.R. (1995) Mutation of the principal sigma factor causes loss of virulence in a strain of the *Mycobacterium tuberculosis* complex. *Proc Natl Acad Sci USA* **92**: 8036–8040.

Coy, M., Doyle, C., Besser, J., and Neilands, J.B. (1994) Site-directed mutagenesis of the ferric uptake regulation gene of *Escherichia coli*. *Biometals* **7**: 292–298.

Curcic, R., Dhandayuthapani, S., and Deretic, V. (1994) Gene expression in mycobacteria: transcriptional fusions based on *xylE* and analysis of the promoter region of the response regulator *mtrA* from *Mycobacterium tuberculosis*. *Mol Microbiol* **13**: 1057–1064.

Deretic, V., Philipp, W., Dhandayuthapani, S., Mudd, M.H., Curcic, R., Garbe, T., *et al.* (1995) *Mycobacterium tuberculosis* is a natural mutant with an inactivated oxidative-stress regulatory gene: implications for sensitivity to isoniazid. *Mol Microbiol* **17**: 889–900.

De Voss, J.J., Rutter, K., Schroeder, B.G., and Barry, C.E. (1999) Iron acquisition and metabolism by mycobacteria. *J Bacteriol* **181**: 4443–4451.

De Voss, J.J., Rutter, K., Schroeder, B.G., Su, H., Zhu, Y., and Barry, C.E. (2000) The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. *Proc Natl Acad Sci USA* **97**: 1252–1257.

Dhandayuthapani, S., Zhang, Y., Mudd, M.H., and Deretic, V. (1996) Oxidative stress response and its role in sensitivity to isoniazid in mycobacteria: characterization and inducibility of *ahpC* by peroxides in *Mycobacterium smegmatis* and lack of expression in *M. aurum* and *M. tuberculosis*. *J Bacteriol* **178**: 3641–3649.

Dussurget, O., Rodriguez, M., and Smith, I. (1996) An *ideR* mutant of *Mycobacterium smegmatis* has derepressed siderophore production and an altered oxidative-stress response. *Mol Microbiol* **22**: 535–544.

Escolar, L., Perez-Martin, J., and Lorenzo, V. (1999) Opening the iron box: transcriptional metalloregulation by the Fur protein. *J Bacteriol* **181**: 6223–6229.

Fiss, E.H., Yu, S., and Jacobs, W.R., Jr (1994) Identification of genes involved in the sequestration of iron in mycobacteria: the ferric exochelin biosynthetic and uptake pathways. *Mol Microbiol* **14**: 557–569.

Glickman, M.S., Cox, J.S., and Jacobs, W.R., Jr (2000) A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of *Mycobacterium tuberculosis*. *Mol Cell* **5**: 717–727.

Gomez, M., and Smith, I. (2000) Determinants of mycobacterial gene expression. In *Molecular Genetics of Mycobacteria.* Hatfull, G.F., and Jacobs, W.R., Jr (eds).

Washington, DC: American Society for Microbiology Press, pp. 111–129.

Gordon, S., Chung, G., and Andrew, P. (1998) The application of bacterial luciferase as a reporter gene in mycobacteria. *Methods Mol Biol* **101**: 235–243.

Hahn, J.S., Oh, S.Y., and Roe, J.H. (2000) Regulation of the *furA* and *catC* operon, encoding a ferric uptake regulator homologue and catalase-peroxidase, respectively, in *Streptomyces coelicolor* A3(2). *J Bacteriol 182*: 3767–3774.

Heym, B., and Cole, S.T. (1992) Isolation and characterization of isoniazid-resistant mutants of *Mycobacterium smegmatis* and *M. aurum*. *Res Microbiol* **143**: 721–730.

Heym, B., Honore, N., Truffot, P.C., Banerjee, A., Schurra, C., van Jacobs, W.J.E.J., *et al.* (1994) Implications of multidrug resistance for the future of short-course chemotherapy of tuberculosis: a molecular study. *Lancet* **344**: 293–298.

Heym, B., Alzari, P.M., Honore, N., and Cole, S.T. (1995) Missense mutations in the catalase-peroxidase gene, *katG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Mol Microbiol* **15**: 235–245.

Heym, B., Stavropoulos, E., Honore, N., Domenech, P., Saint, J.B., Wilson, T.M., *et al.* (1997) Effects of overexpression of the alkyl hydroperoxide reductase AhpC on the virulence and isoniazid resistance of *Mycobacterium tuberculosis*. *Infect Immun* **65**: 1395–1401.

Hill, P.J., Cockayne, A., Landers, P., Morrissey, J.A., Sims, C.M., and Williams, P. (1998) SirR, a novel iron-dependent repressor in *Staphylococcus epidermidis*. *Infect Immun* **66**: 4123–4129.

Johnsson, K., Froland, W.A., and Schultz, P.G. (1997) Overexpression, purification, and characterization of the catalase-peroxidase KatG from *Mycobacterium tuberculosis*. *J Biol Chem* **272**: 2834–2840.

Li, Z., Kelley, C., Collins, F., Rouse, D., and Morris, S. (1998) Expression of *katG* in *Mycobacterium tuberculosis* is associated with its growth and persistence in mice and guinea pigs. *J Infect Dis* **177**: 1030–1035.

McKinney, J.D., Honer zu Bentrup, K., Munoz-Elias, E.J., Miczak, A., Chen, B., Chan, W.T., *et al.* (2000) Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**: 735–738.

Manca, C., Paul, S., Barry, C.R., Freedman, V.H., and Kaplan, G. (1999) *Mycobacterium tuberculosis* catalase and peroxidase activities and resistance to oxidative killing in human monocytes *in vitro*. *Infect Immun* **67**: 74–79.

Middlebrook, G. (1954) Isoniazid-resistance and catalase activities of tubercle bacilli. A preliminary report. *Am Rev Tuberc* **69**: 471–472.

Middlebrook, G., and Cohn, M.L. (1953) Some observations on the pathogenicity of isoniazid-resistant variants of tubercle bacilli. *Science* **118**: 297–299.

Musser, J.M., Kapur, V., Williams, D.L., Kreiswirth, B.N., van Soolingen, D., and van Embden, J.D. (1996) Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and -susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance. *J Infect Dis* **173**: 196–202.

Ortiz de Orue Lucana, D., and Schrempf, H. (2000) The DNA-binding characteristics of the *Streptomyces reticuli* regulator FurS depend on the redox state of its cysteine residues. *Mol Gen Genet* **264**: 341–353.

Pagan-Ramos, E., Song, J., McFalone, M., Mudd, M.H., and Deretic, V. (1998) Oxidative stress response and characterization of the *oxyR–ahpC* and *furA–katG* loci in *Mycobacterium marinum*. *J Bacteriol* **180**: 4856–4864.

Pelicic, V., Reyrat, J.M., and Gicquel, B. (1998) Genetic advances for studying *Mycobacterium tuberculosis* pathogenicity. *Mol Microbiol* **28**: 413–420.

Rouse, D.A., DeVito, J.A., Li, Z., Byer, H., and Morris, S.L. (1996) Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosis*: effects on catalase-peroxidase activities and isoniazid resistance. *Mol Microbiol* **22**: 583–592.

Saint Joanis, B., Souchon, H., Wilming, M., Johnsson, K., Alzari, P.M., and Cole, S.T. (1999) Use of site-directed mutagenesis to probe the structure, function and isoniazid activation of the catalase/peroxidase, KatG, from *Mycobacterium tuberculosis*. *Biochem J* **338**: 753–760.

Sherman, D.R., Sabo, P.J., Hickey, M.J., Arain, T.M., Mahairas, G.G., Yuan, Y., *et al.* (1995) Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. *Proc Natl Acad Sci USA* **92**: 6625–6629.

Sreevatsan, S., Pan, X., Zhang, Y., Deretic, V., and Musser, J.M. (1997) Analysis of the *oxyR–ahpC* region in isoniazid-resistant and -susceptible *Mycobacterium tuberculosis* complex organisms recovered from diseased humans and animals in diverse localities. *Antimicrob Agents Chemother* **41**: 600–606.

van Vliet, A.H., Baillon, M.L., Penn, C.W., and Ketley, J.M. (1999) *Campylobacter jejuni* contains two *fur* homologs: characterization of iron-responsive regulation of peroxide stress defense genes by the PerR repressor. *J Bacteriol* **181**: 6371–6376.

Wilson, T.M., de Lisle, G.W., and Collins, D.M. (1995) Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium bovis*. *Mol Microbiol* **15**: 1009–1015.

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.

Zhang, Y., Garbe, T., and Young, D. (1993) Transformation with *katG* restores isoniazid-sensitivity in *Mycobacterium tuberculosis* isolates resistant to a range of drug concentrations. *Mol Microbiol* **8**: 521–524.

Zou, P., Borovok, I., Ortiz de Orue Lucana, D., Muller, D., and Schrempf, H. (1999) The mycelium-associated *Streptomyces reticuli* catalase-peroxidase, its gene and regulation by FurS. *Microbiology* **145**: 549–559.