Molecular Microbiology (2001) **39**(5), 1174–1185

Mycobacterial FurA is a negative regulator of catalase–peroxidase gene *katG*

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Summary

In several bacteria, the catalase–peroxidase gene *katG* is under positive control by *oxyR*, a transcriptional regulator of the peroxide stress response. The *Mycobacterium tuberculosis* genome also contains sequences corresponding to *oxyR*, but this gene has been inactivated in the tubercle bacillus because of the presence of multiple mutations and deletions. Thus, *M. tuberculosis katG* and possibly other parts of the oxidative stress response in this organism are either not regulated or are controlled by a factor different from OxyR. The mycobacterial FurA is a homologue of the ferric uptake regulator Fur and is encoded by a gene located immediately upstream of *katG*. Here, we examine the possibility that FurA regulates *katG* expression. Inactivation of *furA* on the *Mycobacterium smegmatis* chromosome, a mycobacterial species that also lacks an *oxyR* homologue, resulted in derepression of *katG*, concomitant with increased resistance of the *furA* mutant to H_2O_2. In addition, *M. smegmatis furA*::Km^r was more sensitive to the front-line antituberculosis agent isonicotinic acid hydrazide (INH) compared with the parental *furA*$^+$ strain. The phenotypic manifestations were specific, as the mutant strain did not show altered sensitivity to organic peroxides, and both H_2O_2 and INH susceptibility profiles were complemented by the wild-type *furA*$^+$ gene. We conclude that FurA is a second regulator of oxidative stress response in mycobacteria and that it negatively controls *katG*. In species lacking a functional *oxyR*, such as *M. tuberculosis and M. smegmatis*, FurA appears to be a dominant regulator affecting mycobacterial physiology and intracellular survival.

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Introduction

Mycobacterium tuberculosis is a facultative intracellular pathogen capable of surviving and persisting in phagocytic cells (Armstrong and Hart, 1971; Dannenberg *et al*., 1994). The ability to resist killing by reactive oxygen (Chan *et al*., 1991; Yuan *et al*., 1995; Manca *et al*., 1999) and nitrogen intermediates (Yu *et al*., 1999), prevent phagosomal maturation into the phagolysosome (Armstrong *et al*., 1971; Clemens and Horwitz, 1995; Russell, 1995; Deretic and Fratti, 1999) and avoid detection and elimination by the host's immune system (Pancholi *et al*., 1993; Stenger *et al*., 1998; Mustafa *et al*., 1999) may contribute to the overall potency of this pathogen. In this context, genes and factors involved in protection against oxidative stress are likely to play a role in detoxification of reactive oxygen and nitrogen intermediates encountered upon entry and during residence in infected macrophages. Specifically, the oxidative stress response genes encoding the catalase-peroxidase (*katG*) and the catalytic subunit of alkyl hydroperoxidase reductase (*ahpC*) have been implicated in intracellular survival and persistence of pathogenic mycobacteria in the host (Middlebrook and Cohn, 1953; Morse *et al*., 1954; Mitchison *et al*., 1963; Wilson *et al*., 1995; 1998; Heym *et al*., 1997; Chen *et al*., 1998; Li *et al*., 1998; Manca *et al*., 1999; Cooper *et al*., 2000). In parallel to the potential role in pathogenicity, these and additional determinants have been shown to participate in acquired resistance (Zhang *et al*., 1992; Banerjee *et al*., 1994; Heym *et al*., 1995; Musser, 1995; Rouse and Morris, 1995; Mdluli *et al*., 1998) and innate susceptibility to isonicotinic acid hydrazide (INH) (Deretic *et al*., 1996; Zhang *et al*., 1996).

Understanding factors that contribute to the regulation of oxidative stress response should also aid in the dissection of host–pathogen interactions associated with mycobacterial diseases. In enteric bacteria, the oxidative stress response is mediated by the regulated expression of *katG* and *ahpC* in addition to other factors (Storz and Imlay, 1999). Both genes, as well as several others (Christman *et al*., 1985; Tartaglia *et al*., 1989; Altuvia *et al*., 1994), are under the positive regulation of *oxyR*, a central transcriptional regulator of the peroxide stress response (Christman *et al*., 1989; Storz and Altuvia, 1994). In *M. tuberculosis and* other members of the *M. tuberculosis* complex (*Mycobacterium bovis*, *Mycobacterium africanum and Mycobacterium microti*), the orthologue of *oxyR*

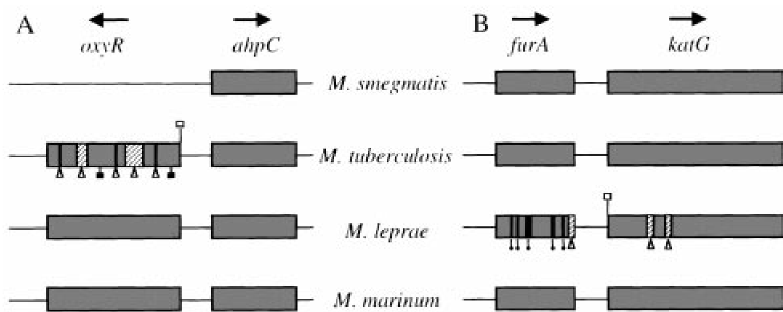

Fig. 1. Genetic organization of the *ahpC–oxyR* (A) and *furA–katG* (B) regions in mycobacteria. Arrows, direction of transcription; open triangles below hatched segments, large deletions; triangles below lines, frameshift mutations; filled balloons, frameshift insertions; open squares, mutations in the start codon; closed squares, nonsense mutations.

has been rendered inactive via multiple mutations (Fig. 1A) (Deretic *et al*., 1995; Sherman *et al*., 1995). Consequently, only limited induction of *katG* and *ahpC* in *M. tuberculosis* can be observed upon exposure to peroxides, possibly contributing to the exquisite sensitivity of *M. tuberculosis* to INH (Deretic *et al*., 1996; Zhang *et al*., 1996). In contrast, *Mycobacterium leprae*, an organism not sensitive to INH, possesses complete *oxyR and ahpC* genes, but has multiple lesions in the *katG* gene (Eiglmeier *et al*., 1997; Nakata *et al*., 1997). Thus, the absence of functional components of the oxidative stress response appears to be a common theme in pathogenic mycobacteria (Deretic *et al*., 1997), albeit such phenomena appear counterintuitive in the context of intracellular survival in infected macrophages (Chan and Kaufmann, 1994). In addition to the partial elimination of elements of the oxidative stress response in the two major human mycobacterial pathogens, *M. tuberculosis and M. leprae*, several non-pathogenic mycobacterial species also differ from the enterobacterial paradigm of oxidative stress response. For example, the fast-growing species *Mycobacterium smegmatis* displays induced expression of at least nine proteins (Dhandayuthapani *et al*., 1996) in response to peroxide stress, including *katG* (Sherman *et al*., 1995) and *ahpC* (Sherman *et al*., 1995; Dhandayuthapani *et al*., 1996), although it lacks an *oxyR* equivalent (Fig. 1A). Furthermore, in *Mycobacterium marinum*, a pathogenic non-tuberculous species (Falkinham, 1996) that is relatively closely related to *M. tuberculosis* (Rogall *et al*., 1990), *oxyR* does not regulate expression of *katG* although it controls *ahpC* (Pagan-Ramos *et al*., 1998). Thus, it seems plausible that genes and mechanisms different from those previously identified in Enterobacteriaceae may control aspects of oxidative stress response in mycobacteria.

Apart from *oxyR*-dependent regulation of oxidative stress response, many organisms couple the expression of oxidative stress genes with iron metabolism, principally via the ferric uptake regulator Fur. Fur or Fur homologues regulate genes induced in response to oxidative stress, including *sodA* (Niederhoffer *et al*., 1990; Tardat and Touati, 1993) and *sodB* (Niederhoffer *et al*., 1990; Dubrac *et al*., 2000) encoding Mn^{2+} and Fe^{2+} superoxide dismutases (SOD), the 8-hydroxyguanine endonuclease gene (Lee *et al*., 1998), catalase and peroxidase genes (Hassett *et al*., 1997; Bsat *et al*., 1998; van Vliet *et al*., 1998; 1999; Baillon *et al*., 1999), alkyl hydroperoxidase genes (Bsat *et al*., 1998; van Vliet *et al*., 1998; 1999), the *soxRS* genes (Zheng *et al*., 1999) and even the *oxyR* gene (Zheng *et al*., 1999). Mycobacteria also appear to couple expression of oxidative stress genes with iron metabolism. For example, inactivation of *ideR*, encoding an orthologue of the iron-responsive *Cornybacterium diphtheriae* repressor DtxR, renders *M. smegmatis* more sensitive to H_2O_2 as a result of decreased KatG and Mn^{2+} SOD activities (Dussurget *et al*., 1996).

The presence of a *fur*-like gene, *furA*, immediately upstream of *katG* in several mycobacterial species including *M. tuberculosis*, *M. leprae* and *M. marinum* (Fig. 1B) led us to propose that a subset of oxidative stress response genes may be regulated by FurA (Deretic *et al*., 1997; Pagan-Ramos *et al*., 1998). To investigate further the potential role of *furA* in the regulation of oxidative stress genes in mycobacteria, we characterized the *furA* gene of *M. smegmatis* and tested its regulatory role in the oxidative stress response. We present evidence demonstrating that *furA* negatively regulates *katG* expression. Inactivation of *furA* on the chromosome of *M. smegmatis* increased both the resistance of this organism to H_2O_2 and its susceptibility to INH. These observations have implications for the virulence of mycobacteria in the context of the peculiar evolutionary events that lead to a deregulation or partial elimination of oxidative stress response in pathogenic mycobacteria.

Results

The furA *and* katG *genes are genetically linked in* M. smegmatis *and other mycobacteria*

The *furA* gene is located immediately upstream of *katG* in *M. tuberculosis*, *M. leprae* and *M. marinum* (Deretic *et al*., 1997; Pagan-Ramos *et al*., 1998). To determine whether the *furA and katG* linkage is also conserved in

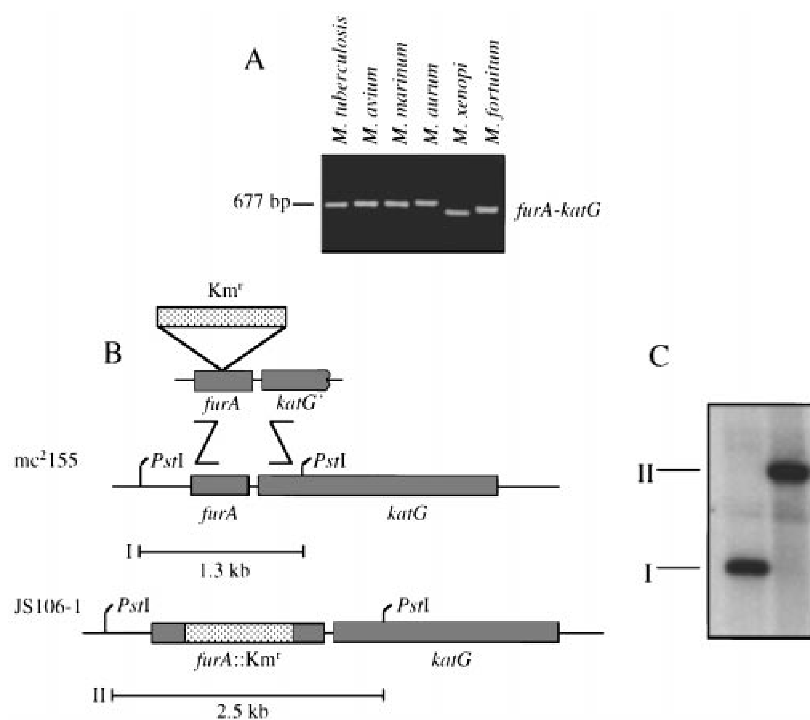

Fig. 2. Conservation of *furA–katG* linkage in mycobacterial species and inactivation of the *furA* gene in *M. smegmatis* mc^{2}155 via homologous recombination.
A. Two degenerate primers, FurAZooL and FurAZooR (see *Experimental procedures*), corresponding to conserved regions of *furA and katG* were used to amplify the corresponding regions from *M. tuberculosis*, *M. avium*, *M. marinum*, *M. aurum*, *M. xenopi* and *M. fortuitum*. The identity of PCR fragments as *furA–katG* was confirmed by sequencing.
B. Inactivation of the *furA* gene in *M. smegmatis* mc^{2}155 via homologous recombination. The *furA* gene from *M. smegmatis* was disrupted by allelic exchange using a linear DNA fragment containing the *furA* gene with a Km^r cassette insertion.
C. Southern blot hybridization analysis using *Pst*I-digested chromosomal DNA and a *furA*-specific radiolabelled probe. I, 1.3 kb *Pst*I fragment from wild-type *M. smegmatis* mc^{2}155; II, 2.5 kb *Pst*I fragment from *furA*::Km^r isolate JS106-1 (see scheme in B).

M. smegmatis, we cloned and sequenced the region immediately upstream of *katG* in *M. smegmatis* mc^{2}155. An open reading frame (ORF; GenBank accession number AF012631) encoding a polypeptide of 126 amino acids with high homology to FurA from other mycobacterial species was present immediately upstream of *katG*. *M. smegmatis* FurA was 65% identical to *M. tuberculosis* FurA and 57% identical to *M. marinum* FurA (data not shown). Additional polymerase chain reaction (PCR) analysis of diverse mycobacterial species using degenerate primers based on conserved regions of *furA and katG* also detected the *furA–katG* linkage in *Mycobacterium avium*, *Mycobacterium aurum*, *Mycobacterium xenopi* and *Mycobacterium fortuitum*, in addition to *M. tuberculosis and M. marinum* (Fig. 2A). The corresponding DNA fragments containing partial *furA and katG* genes were subjected to DNA sequencing and their identity confirmed (GenBank accession numbers AF092559, AF092558 and AF092560). Thus, the *furA–katG* linkage was found to be conserved in all six of the mycobacteria species analysed.

Gene replacements with furA*::Km^r in* M. smegmatis *and analysis of phenotypic effects on sensitivity to oxidants*

The ubiquitous presence in mycobacteria of *furA* immediately upstream of the catalase–peroxidase gene *katG* led us to investigate whether the expression of oxidative stress response genes in *M. smegmatis* was regulated by *furA*. To this end, we inactivated *furA* on the chromosome of *M. smegmatis* mc^{2}155. Using a linear DNA fragment carrying a disrupted copy of *furA*, *furA*::Km^r mutants were generated in *M. smegmatis* by gene replacement via homologous recombination (Fig. 2B). Southern blot analysis performed on one such recombinant, JS106-1, confirmed that a true gene replacement of *furA*$^+$ with *furA*::Km^r had occurred (Fig. 2C). To determine whether inactivation of *furA* in *M. smegmatis* grown in 7H9 medium had phenotypic effects on oxidative stress, we examined the susceptibility of JS106-1 to peroxides (Table 1). Somewhat unexpectedly, JS106-1 (*furA*::Km^r) was significantly more resistant to H_2O_2 than the parental *furA*$^+$ strain *M. smegmatis* mc^{2}155 (Table 1, rows A and B; $P = 0.0001$, ANOVA). The decreased sensitivity to H_2O_2 was a direct result of the *furA*::Km^r alteration, because complementation of the *furA*::Km^r mutation in JS106-1 by the introduction of pMsFurA carrying a wild-type *furA*$^+$ gene (strain JS106-1 [pMsFurA]) reversed the increased resistance to H_2O_2 (Table 1, rows B and C; $P = 0.0001$, ANOVA). When sensitivity to cumene hydroperoxide (CHP) was tested, no significant differences in susceptibility to this organic peroxide were observed between mc^{2}155, JS106-1 and JS106-1 [pMsFurA] strains (Table 1, rows A–C). In contrast, an *ahpC*::Km^r mutant of *M. smegmatis* mc^{2}155, VD1865-6, showed increased sensitivity to CHP (Table 1, row D; $P = 0.0001$, ANOVA). These results are consistent with the interpretation that inactivation of *furA* derepresses a system specifically involved in detoxification of hydrogen peroxide but not organic peroxides.

Table 1. Sensitivity[a] of *M. smegmatis* to peroxides and INH.

Row	Strain[b]	Genotype	H_2O_2	CHP	INH
A	mc^2155	*furA*$^+$	31.3 ± 0.3	31.7 ± 0.3	20.3 ± 0.3
B	JS106-1	*furA*::Km^r	26.7 ± 0.3	31.7 ± 0.3	50.3 ± 0.6
C	JS106-1 [pMsFurA]	*furA*::Km^r [*furA*$^+$]	39.7 ± 0.3	31.3 ± 0.3	31.7 ± 0.3
D	VD1865-6	*ahpC*::Km^r	35.0 ± 0.6	38.7 ± 0.3	36.3 ± 0.3

a. Values represent the mean diameter ± SE (in mm) of zones of inhibition (experiments performed in triplicate) from strains grown in 7H9 medium. Filter disks (quarter-inch diameter) were soaked with 10 μl of 2% H_2O_2, 2% CHP or 1 mg ml^{-1} INH. Results were read 3 days after plating.
b. JS106-1, JS106-1 [pMsFurA] and VD1865-6 are isogenic derivatives of *M. smegmatis* mc^2155.

The furA *gene is a negative regulator of* katG *expression*

The linkage between *furA and katG* in all mycobacteria and changes in sensitivity to H_2O_2 in the *M. smegmatis furA* mutant are suggestive of a role for *furA* in *katG* expression. To determine whether *furA* regulates *katG* expression, we compared steady-state levels of KatG in the *M. smegmatis furA*::Km^r mutant JS106-1 and its *furA*$^+$ parent mc^2155. In the *furA*::Km^r background, KatG levels were markedly increased (Fig. 3A, lanes 1 and 2). A quantitative analysis of the steady-state KatG levels indicated a 4.2-fold increase in KatG in JS106-1 relative to mc^2155 (Fig. 3A; $P = 0.0001$, ANOVA). The overexpression of *katG* observed in JS106-1 was a direct result of the disruption of the *furA* gene, as plasmid pMsFurA (*furA*$^+$) complemented the JS106-1 phenotype (Fig. 3A, lanes 2 and 3; $P = 0.0001$, ANOVA). We attribute the reduction in KatG levels in JS106-1 [pMsFurA] beyond those observed in the *furA*$^+$ wild-type strain mc^2155 (Fig. 3A, lanes 1 and 3) to multicopy effects of plasmid-borne *furA*$^+$. The less than wild-type levels of KatG in JS106-1 [pMsFurA] (Fig. 3A, lanes 1 and 3) were also in keeping with the hypersensitivity of the complemented strain to H_2O_2 (Table 1, rows A and C). Next, we investigated whether *furA*, in addition to KatG, affected steady-state levels of AhpC. When strains mc^2155, JS106-1 and JS106-1 [pMsFurA] were tested, no significant differences in AhpC amounts were observed (Fig. 3B). These observations were also in keeping with the lack of detectable changes in susceptibility to CHP between mc^2155, JS106-1 and JS106-1 [pMsFurA] (Table 1, rows A–C). In conclusion, the data presented indicate that FurA is a negative regulator of *katG* but not of *ahpC* in *M. smegmatis*.

Analysis of the role of furA *in* katG *and* ahpC *expression in response to oxidants and iron availability*

We next investigated whether *furA* affected steady-state levels of KatG and AhpC upon exposure to oxidants. The absence of *furA* did not significantly affect KatG levels in

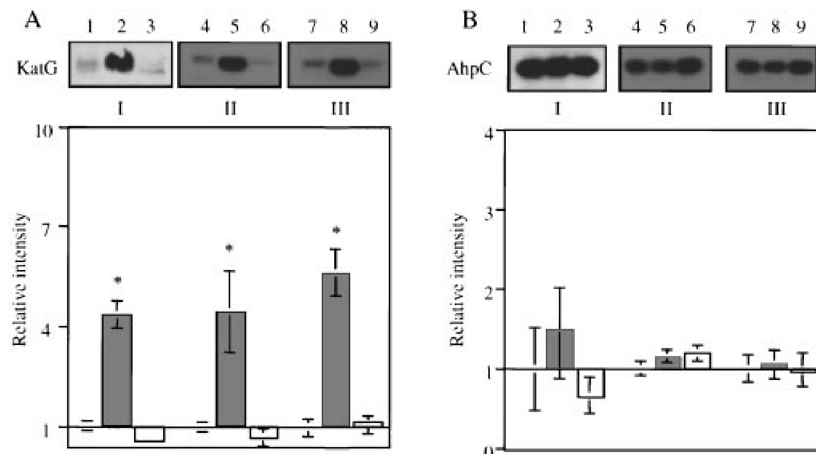

Fig. 3. Steady-state KatG and AhpC levels in *M. smegmatis* strains grown in Sauton's minimal medium containing different concentrations of iron. Western blot analysis and band intensity quantification were performed using total protein extracts (5 μg) from wild-type mc^2155 (*furA*$^+$ lanes 1, 4 and 7), JS106-1 (*furA*::Km^r; lanes 2, 5 and 8), and JS106-1 [pMsFurA] (*furA*::Km^r [p*furA*$^+$]; lanes 3, 6 and 9) and antibodies that recognize mycobacterial KatG (A) and AhpC (B). Strains were grown in minimal medium supplemented with 0.5 μM Fe^{2+} (set I: lanes 1–3), 5 μM Fe^{2+} (set II: lanes 4–6) or 70 μM Fe^{2+} (set III: lanes 7–9). Western blots from representative gels are shown in each case. Graphs represent quantitative analyses of band intensities (see *Experimental procedures*) performed on independent triplicate cultures. Relative intensities of KatG and AhpC were normalized against wild-type mc^2155 levels and expressed as mean ± SE values. Statistical analysis was performed on mean relative intensity under each condition tested. *, $P < 0.005$ (ANOVA); mc^2155 relative to JS106-1.

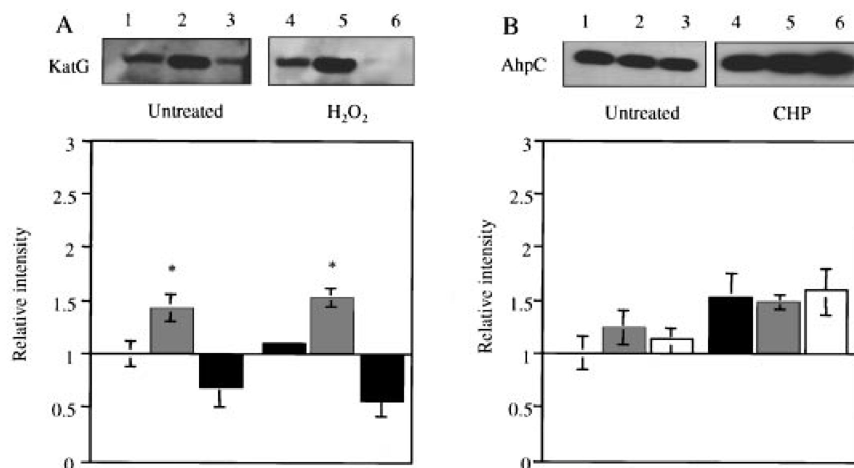

Fig. 4. Steady-state KatG and AhpC levels in *M. smegmatis* strains grown in 7H9 and exposed to H_2O_2 and cumene hydroperoxide (CHP). Western blot analysis and band intensity quantification were performed using total protein extracts (25 μg) from wild-type mc^{2}155 (*furA*$^+$ lanes 1 and 4), JS106-1 (*furA*::Km^r; lanes 2 and 5) and JS106-1 [pMsFurA] (*furA*::Km^r [p*furA*$^+$]; lanes 3 and 6) using KatG (A) and AhpC (B) antibodies. Western blots from one representative gel are shown. Relative intensities of KatG and AhpC are mean ± SE values from at least three independent cultures. Statistical analysis was performed on mean relative intensity under each condition tested. *, $P < 0.05$ (ANOVA); mc^{2}155 relative to JS106-1.

cells treated with subinhibitory concentrations (125 μM) of H_2O_2 (Fig. 4A). This concentration of H_2O_2 was previously determined to provide optimal induction analysis (Dhandayuthapani *et al*., 1996). These results suggest that steady-state KatG levels do not change significantly in *M. smegmatis* upon H_2O_2 treatment at concentrations of this agent previously shown to induce *ahpC and* other polypeptides in this organism (Sherman *et al*., 1995; Dhandayuthapani *et al*., 1996). Furthermore, the derepression of *katG* in the *furA*::Km^r mutant was independent of peroxide stimulation (Fig. 4A). We also examined steady-state levels of AhpC after treatment with subinhibitory concentrations (125 μM) of the organic peroxide CHP. This concentration of CHP was previously determined to be optimal for induction studies (Dhandayuthapani *et al*., 1996). Treatment of mc^{2}155, JS106-1 and JS106-1 [pMsFurA] with CHP resulted in an ≈1.5-fold increase in steady-state levels of AhpC over untreated wild-type levels (Fig. 4B; $P = 0.0352$, ANOVA). However, as expected, this increase was independent of *furA*, as higher levels of AhpC were achieved in both *furA*$^+$ and *furA*::Km^r mutant cells (Fig. 4B, lanes 2 and 5). These results are also consistent with the interpretation that *furA* does not affect steady-state levels of AhpC and is not involved in AhpC increases upon exposure to peroxides.

As the mycobacterial FurA shows strong similarities to the iron uptake regulator Fur and other Fur-like elements (Deretic *et al*., 1997; Pagan-Ramos *et al*., 1998), we next tested whether *furA* regulated steady-state levels of KatG in response to iron. When mc^{2}155, JS106-1 and JS106-1 [pMsFurA] were grown in conditions of low (0.5 μM), intermediate (5.0 μM) or high (70 μM) iron (Wong *et al*., 1999), no significant differences in KatG levels were observed in the context of *furA* repression of *katG* (Fig. 3A; set I, 0.5 μM Fe^{2+} set II, 5.0 μM Fe^{2+} set III, 70 μM Fe^{2+}). In keeping with these observations, sensitivities to H_2O_2 of *M. smegmatis* mc^{2}155 and its derivatives JS106-1 and JS106-1 [pMsFurA] remained unaffected when bacteria were grown in Sauton's minimal medium supplemented with various concentrations of iron (data not shown). Owing to similarities with the ferric uptake regulator Fur, we also examined whether inactivation of *furA* affected the production of siderophores in *M. smegmatis*, a known iron-regulated system (Dussurget *et al*., 1996). No differences in siderophore secretion between the *furA*$^+$, *furA*::Km^r and an *ideR*::Km^r strain were observed on CAS medium supplemented with 0.5 μM Fe^{2+} (6 mm secretion zones; data not shown). However, on CAS medium supplemented with 70 μM Fe^{2+} (Fig. 5A), high iron repressed siderophore secretion in wild-type *M. smegmatis* mc^{2}155 (0 mm secretion zone), but not in the *ideR* mutant (6 mm secretion zone). The siderophore repression by iron also remained unaltered in the *furA*::Km^r mutant strain JS106-1 (Fig. 5A). Thus, in contrast to IdeR, FurA most probably plays no major role in the aspect of iron regulation in *M. smegmatis* that involves siderophore secretion.

Comparison of the effects of furA *inactivation on KatG and KatE expression levels*

M. smegmatis synthesizes two enzymes with catalase activity: KatG, which functions as a catalase and peroxidase (Zhang *et al*., 1992; Heym *et al*., 1993); and KatE,

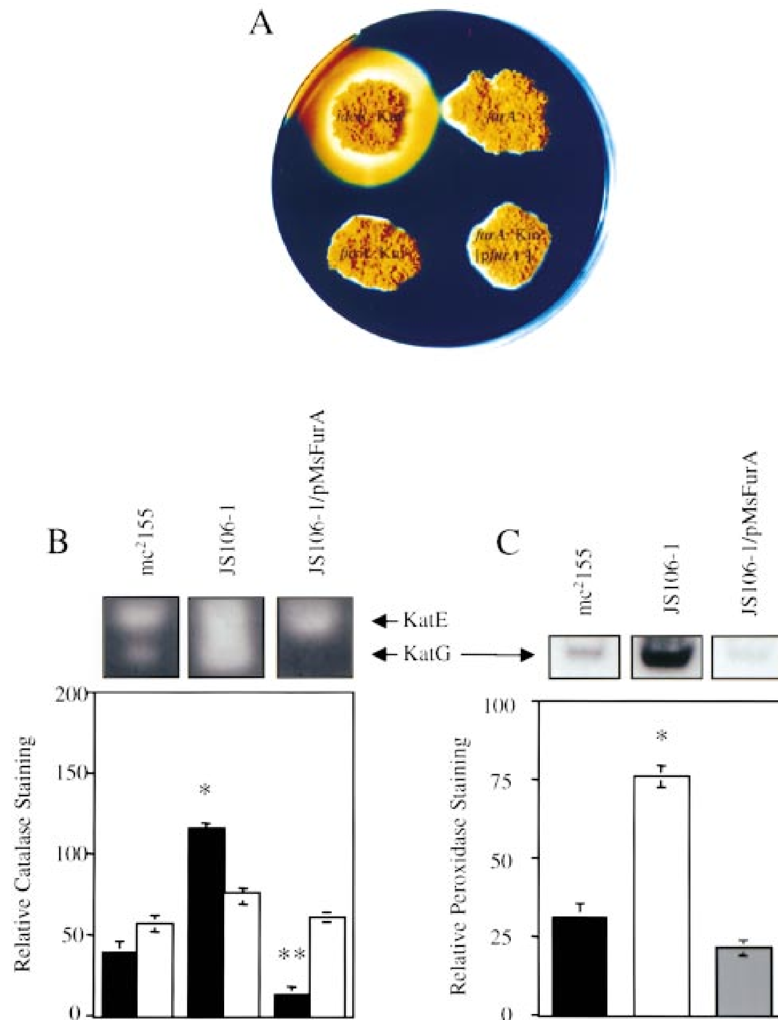

Fig. 5. Comparison of siderophore secretion and peroxidase and catalase activity zymograms in *furA*$^+$ and *furA* mutants from *M. smegmatis*.
A. Siderophore secretion from wild-type strain mc^{2}155 (*furA*$^+$), JS106-1 (*furA*::Km^r), JS106-1 [pMsFurA] (*furA*::Km^r [*furA*$^+$]) and SM3 (*ideR*::Km^r) was analysed on CAS medium containing 70 μM Fe^{2+}. Derepression of siderophore secretion produces a yellow halo. Secretion zones were measured from the colony edge.
B and C. Relative peroxidase and catalase activities of total protein extracts from mc^{2}155, JS106-1 and JS106-1 [pMsFurA] after activity gel staining. Total protein extract (50 μg) was loaded and run on non-denaturing polyacrylamide gels and stained for catalase (B) or peroxidase (C) activities as described in *Experimental procedures.* In (B), KatE activity is the upper, slower migrating band (open bars), and KatG activity is the faster migrating, lower band (filled bars). Activity staining from one representative gel is shown. Relative staining is the mean ± SE from at least three independent cultures. Statistical analysis was performed on mean values from relative catalase or peroxidase activity staining between mc^{2}155 and either JS106-1 or JS106-1 [pMsFurA]. *, $P < 0.0001$ (ANOVA); mc^{2}155 relative to JS106-1; **, $P < 0.05$ (ANOVA); mc^{2}155 relative to JS106-1 [pMsFurA].

with catalase activity only (Bartholomew, 1968; Dussurget *et al*., 1996). Because inactivation of *furA* increased the expression of *katG* (Figs 3 and 4) and altered the susceptibility of *M. smegmatis* to H_2O_2 (Table 1), we next examined whether *furA* also regulated the expression of the *katE* gene, encoding the heat-stable catalase. When protein extracts from wild-type mc^{2}155, JS106-1 (*furA*::Km^r) and JS106-1 [pMsFurA] (*furA*::Km^r/*furA*$^+$) were subjected to zymogram analysis for catalase activity, no significant differences were observed in *katE*-encoded catalase activity (Fig. 5B). This was in contrast to a significant increase in *katG*-encoded catalase activity observed in JS106-1 compared with wild-type mc^{2}155 (Fig. 5B; $P < 0.0001$, ANOVA). The increase in *katG*-encoded catalase activity stain was a direct result of *furA* disruption, as complementation of JS106-1 with pMsFurA reversed the relative increase in KatG catalase staining back below wild-type levels (Fig. 5B; $P = 0.0175$). These results are consistent with the relative increase and decrease in steady-state KatG levels observed by Western blot analysis in JS106-1 and JS106-1 [pMsFurA] strains respectively (Figs 3A and 4A). The increase in *katG* expression in JS106-1 also resulted in increased peroxidase activity staining compared with wild-type mc^{2}155 (Fig. 5C; $P < 0.0001$). These results suggest that, unlike *katG*, *katE* is most probably not regulated by *furA*. Furthermore, the increase in *katG* expression after disruption of *furA* results in an increase in both catalase and peroxidase activities of KatG, but does not indirectly alter KatE catalase activity.

Sensitivity to INH and furA

Inactivation of *furA* decreases the susceptibility of *M. smegmatis* to H_2O_2 (Table 1) and increases steady-state levels of KatG (Figs 3A and 4A). As KatG has been postulated to activate the prodrug INH, transforming it intracellularly into an active form (Wengenack *et al*., 1998;

Lei *et al*., 2000), we tested whether the loss of *katG* repression in the *furA*::Km^r mutant would also affect the susceptibility of *M. smegmatis* to INH when grown in 7H9 medium. When JS106-1 (*furA*::Km^r) was compared with mc^{2}155 (*furA*$^+$), a significant increase in susceptibility to INH was observed (Table 1, rows A and B; $P = 0.0001$, ANOVA). Introduction of the complementing plasmid pMsFurA into JS106-1, which reduced *katG* expression to below wild-type levels (Figs 3A and 4A), partially reversed the INH sensitivity phenotype (Table 1, rows A–C; $P = 0.0001$). The inability to complement the INH phenotype fully (Table 1, rows A and C), in contrast to the full complementation of KatG levels (Figs 3A and 4A) and H_2O_2 sensitivity (Table 1), suggests that *furA* may affect or regulate additional genes that respond to multicopy complementation differently from *katG*. Alternatively, excessive repression of *katG* may render *M. smegmatis* increasingly susceptible to some aspects of INH antimycobacterial action that depend on the generation of reactive oxygen intermediates (Shoeb *et al*., 1985) normally detoxified by KatG. Consistent with a lack of effect of iron on KatG activity (Fig. 3A), no additional changes in INH susceptibility were observed in mc^{2}155, JS106-1 and JS106-1 [pMsFurA] when bacteria were assayed under conditions of low, intermediate or high iron (data not shown).

Discussion

In this work, a regulatory role has been established for the mycobacterial *furA* gene. The data presented show that FurA acts as a negative regulator of *katG* in *M. smegmatis*. Although, at this stage, it cannot be stated definitively whether the regulation of *katG* by FurA is direct or indirect, the derepression of *katG* as a result of insertional inactivation of *furA* cannot be attributed to a polar effect on the downstream *katG* gene based on the following considerations. (i) If the insertion were polar, KatG levels would have been reduced and not increased in the mutant, contrary to our observations. (ii) More importantly, the chromosomal mutation in *furA* was complemented *in trans and* its phenotypic consequences reversed by the introduction into the *furA*::Km^r mutant of a plasmid-borne *furA*$^+$. (iii) The genetic complementation also rules out the possibility of increased *katG* expression resulting from promoter activity from the Km^r cassette reading through the *katG* gene.

Based on the invariant *furA*–*katG* linkage in mycobacteria, as reported in *M. tuberculosis*, *M. leprae* and *M. marinum* (Deretic *et al*., 1997; Pagan-Ramos *et al*., 1998) and further extended in this work to include *M. smegmatis*, *M. avium*, *M. aurum*, *M. fortuitum* and *M. xenopi*, it is likely that the regulatory relationships between *furA and katG* are universal in mycobacteria. These relationships may potentially be extended to other actinomycetes, as a similar organization has been reported in *Streptomyces reticuli* (Zou *et al*., 1999) and *Streptomyces coelicolor* (Hahn *et al*., 2000). The regulation of catalase genes by *fur* homologues has also been reported even when the genes are not linked in a number of bacteria including *Bacillus subtilis* (Bsat *et al*., 1998), *Pseudomonas aeruginosa* (Hassett *et al*., 1997) and *Campylobacter jejuni* (van Vliet *et al*., 1998; 1999). Iron-related regulation of the catalase–peroxidase gene *katG* has been observed previously in *M. smegmatis* (Dussurget *et al*., 1996). However, inactivation of *ideR* affects *katG* in a different way from *furA*, as steady-state levels of KatG are reduced in *ideR* mutant cells (Dussurget *et al*., 1996), whereas *furA* inactivation increases KatG levels. The molecular mechanism responsible for *katG* regulation by IdeR is not known and could be indirect, potentially via changing intracellular iron concentrations or other downstream events caused by the elevated expression of siderophores in *ideR* mutant cells. Indirect evidence for the role of iron in the regulation of a closely related catalase–peroxidase gene by a *fur* homologue has been demonstrated in the case of *furS* regulation of *cpeB* in *S. reticuli* (Zou *et al*., 1999). However, these studies were carried out using plasmid constructs in lieu of a bona fide *furS* chromosomal mutant. In addition, iron-mediated (as well as Ni^{2+}, Mn^{2+} and Zn^{2+}) regulation of *catC* by *furA* has been demonstrated in the closely related species *S. coelicolor* (Hahn *et al*., 2000). Although we have not found evidence in support of a role for exogenous iron in the regulation of *katG* by *furA*, this does not preclude the possibility that intracellular iron or another redox active transition metal may affect *katG* expression. Precedents for such relationships have been observed in other organisms, including the manganese-dependent (in addition to iron) regulation of *katA* by PerR in *B. subtilis* (Bsat *et al*., 1998) and the zinc-dependent regulation exhibited by Zur in *Escherichia coli* (Patzer *et al*., 1998). Regardless of the specifics of the potential metal involved or other type of redox sensing by FurA, it appears that regulation of the oxidative stress response by Fur homologues is a widespread mechanism in mycobacteria and other organisms.

The biological properties of strains with inactive *furA* suggest several intriguing phenotypic consequences with broader implications for the physiology and pathogenic characteristics of mycobacteria. First, the *furA*::Km^r strain displays higher resistance to hydrogen peroxide, thus representing an unexpected improvement upon the wild-type strain in detoxification of H_2O_2. Secondly, another notable change is the increased susceptibility of the *furA*::Km^r strain to INH. Both these phenomena can be explained, at least in part, by the increased levels of KatG

and the resulting increase in total catalase and peroxidase activities.

The finding that *furA* regulates the expression of *katG* in *M. smegmatis* potentially sheds light on some of the curious aspects of the evolution of the pathogenic mycobacteria. For example, *M. leprae* has an inactivated *furA* gene (Deretic *et al*., 1997; Pagan-Ramos *et al*., 1998), a phenomenon that, at least at some stage in the speciation of this pathogen, could have enhanced its ability to detoxify peroxides and perhaps survive in the host. Similar selective pressures might have existed in *M. tuberculosis* as elimination of functional *oxyR* took place. The effects of the deregulation of oxidative stress response in these organisms must have provided some selective advantage, as only the frank human pathogens *M. leprae and M. tuberculosis* have undergone such irreversible changes, in sharp contrast to other opportunistic or non-pathogenic mycobacteria.

Experimental procedures

Bacterial strains, media and growth conditions

JS106-1(*furA*::Km^r), VD1865-6 (*ahpC*::Km^r) (Zhang *et al*., 1996) and SM3 (*ideR*::Km^r) (Dussurget *et al*., 1996) are isogenic derivatives of *M. smegmatis* mc^{2}155 (Snapper *et al*., 1990). All transformations performed in *E. coli* were in DH5α. *M. smegmatis* was grown in Middlebrook 7H9 broth or 7H10 agar (Difco) supplemented with ADC (10% bovine serum albumin fraction V, dextrose and sodium chloride), 0.2% glycerol and 0.05% Tween 80 or in a modified Sauton minimal medium (Dussurget *et al*., 1996). When required, 0.75% noble agar (Difco) was added to 7H9 or Sauton media for soft agar. For preparation of Sauton, glassware was soaked with 0.2 M HCl and media treated with 5.0 g l^{-1} Chelex 100 resin (Bio-Rad) to remove metal contaminants before the addition of trace elements and the iron source, ferrous ammonium citrate. *E. coli* was grown in Luria–Bertani (LB) medium (Difco). When required, kanamycin sulphate (Km; Sigma) and hygromycin B (Hyg; Boehringer Mannheim) were added at 25 μg ml^{-1} and 50 μg ml^{-1}, respectively, for *M. smegmatis* or 50 μg ml^{-1} and 200 μg ml^{-1}, respectively, for *E. coli*. For exposure of *M. smegmatis* to peroxides, strains were grown in 7H9 to an optical density at 600 nm (OD_{600}) of 0.5, aliquoted into 5 ml cultures and exposed to 125 μM H_2O_2 or 125 μM CHP (Sigma) for 1 h in a 37°C shaker. Detection of siderophore secretion was performed on CAS medium (Dussurget *et al*., 1996).

Cloning and recombinant DNA techniques

Inverse PCR was used to clone the region upstream of *katG* in *M. smegmatis* based on the sequence of the 5′ end of *M. smegmatis katG* (Billman-Jacobe *et al*., 1996). *M. smegmatis* genomic DNA (2.5 μg) was digested to completion with *Sph*I, purified by Qiaex II (Qiagen) and self-ligated in a 25 μl reaction mixture. Inverse PCR was carried out with 5 μl of the ligation reaction mixture and primers specific to the known portion of *M. smegmatis katG*, Msm-fur2 (5′-CCGGCGGGTTTCGGCCGCATC-3′), and a small 5′ region immediately upstream of *katG*, Msm-fur1 (5′-TTCCTTTCGGGAGTGGTGAAT-3′). A single band corresponding to an 800 bp PCR product was cloned into pCR2.1 (Invitrogen) and sequenced to obtain the complete nucleotide sequence of *M. smegmatis furA*. A *Sac*I-linearized fragment containing *furA*::Km^r from pSM243/Ms*furA*::Km^r was used for the disruption of *furA* in *M. smegmatis* mc^{2}155 by allelic exchange. pSM243/Ms*furA*::Km^r was constructed as follows: primers Ms-fur10A (5′-ACGAGCTCTGCAGAAGGATCCAC-TGAAATTCGATGC-3′; underlined sequence *Sac*I site) and Ms-fur11 (5′-CTACTAGC TAGCCAGCAGACTAGTGTTGT-CGCCGACGCGGATTCGTAGCG-3′; underlined sequence *Spe*I site), and primers Msfur-12 (5′-CTGCTGGCTAGCTA-GTAGACTAGTGTGGTCTGCCGCGCGTGCGGCGACATC-3′; underlined sequence *Spe*I site) and Msfur-13A (5′-GGTGAGCTCCCACTCGTTGCCGTACAGGATCCCAG-3; underlined sequence *Sac*I site) were used to PCR amplify fragments containing the upstream sequence and 5′ half, and 3′ half and downstream sequence, respectively, of *M. smegmatis furA*. These fragments were ligated together, digested with *Sac*I and cloned into the *Sac*I site of pSM243 (provided by Dr I. Smith). An *Nhe*I–*Spe*I fragment encoding the Km^r cassette from pMV206 (Stover *et al*., 1991) was subsequently cloned into the engineered *Spe*I site present in *furA*, resulting in pSM243/Ms*furA*::Km^r. DNA from pSM243/Ms*furA*::Km^r was linearized with *Sac*I, gel purified to enrich for fragments containing *M. smegmatis furA*::Km^r and electroporated into *M. smegmatis* mc^{2}155 as described previously (Jacobs *et al*., 1991). Transformants resulting from double cross-over to exchange *furA*$^+$ for *furA*::Km^r in *M. smegmatis* mc^{2}155 were selected on 7H10 medium containing Km and screened by PCR using primers Ms-fur1 and Ms-fur5 (5′-CCGAGGCCGTCGGAGGAA-3) that flank the Km^r cassette. Plasmid pMsFurA was used for complementation of *furA*::Km^r in *M. smegmatis* JS106-1 and was constructed as follows: a DNA fragment containing *furA* was PCR amplified from *M. smegmatis* mc^{2}155 using primers Ms-fur5 and Ms-fur9 (5′-CTTCTGCAGGATCTTCAGATT-GAGCTGATT-3′) and cloned into pCR2.1. A *BamH*I–*Pst*I fragment containing *furA* was then ligated into the *BamH*I–*Pst*I-digested *E. coli*–mycobacterium shuttle vector pOLYG (Hyg^r) to create pOLYG/Ms*furA*. Finally, the *Xba*I–*Nhe*I-digested *xylE* reporter gene from pHSX-1 (Curcic *et al*., 1994) was cloned into the *Xba*I polylinker site of pOLYG/Ms*furA* to create pMsFurA. JS106-1 transformants containing pMsFurA were selected on 7H10 medium containing Hyg and screened for the presence of *xylE*, detected as yellow colonies upon spraying with 100 mM catechol (Curcic *et al*., 1994). For the amplification of *furA* from other mycobacterial species, two degenerate primers were used: FurAZooL [5′-GTCGGCGACAACCACCACCAC(AG)T(CG)GT(CG)ACG-3′) and FurAZooR [5′-GCCA(CG)AGCAG(CG)CGGCG(CG)G-CCTTCAGT(AG)CG-3′]; residues in parenthesis indicate degenerate positions.

DNA extraction and Southern analysis

Mycobacterial genomic DNA was prepared as described previously (Jacobs *et al*., 1991). For Southern analysis, 4 μg

of genomic DNA was digested overnight with *Pst*I (Gibco BRL), separated by electrophoresis on a 0.8% agarose gel, transferred onto a Duralon-UV membrane (Stratagene) and used in subsequent high-stringency hybridization and washing steps (Pagan-Ramos *et al*., 1998). A *furA*-specific probe from *M. smegmatis* was generated by random-primed labelling (Gibco BRL) with [α-^{32}P]-dCTP (3000 Ci mmol^{-1}; NEN Dupont) using PCR products generated with oligonucleotides Ms-fur1 and Ms-fur5.

Zone inhibition assays

A modified disc inhibition assay (Rosner, 1993) was used to determine the relative sensitivities of *M. smegmatis* derivatives to H_2O_2, CHP and INH. *M. smegmatis* strains were grown to mid-exponential phase ($OD_{600} = 0.5$) in 7H9 or Sauton media containing low (0.5 μM), moderate (5 μM) or high (70 μM) Fe^{2+}. A sample of 0.2 ml of cells was added to 3 ml of tempered 7H9 or iron-supplemented Sauton soft agar, poured onto the respective agar plates and allowed to solidify. Sterile quarter-inch-diameter BBL blank paper disks (Fisher) were added to the centre of bacteria-overlaid plates and saturated with 10 μl of a 2% solution of H_2O_2 or CHP or a 1 mg ml^{-1} solution of INH. Plates were incubated for 3 days at 37°C before zones of growth inhibition were measured.

Immunoblot analysis

Crude cell extracts of strains grown to mid-exponential phase ($OD_{600} = 0.5$) were prepared by homogenization with a mini bead beater and 0.1 mm zirconia beads (Biospec Products) for 1 min. Cell debris and beads were removed by centrifugation, and supernatants were measured for total protein. Aliquots of 25 μg (from 7H9-grown bacteria) or 5 μg (from bacteria grown in Sauton's medium) of total protein from *M. smegmatis* derivatives were separated on SDS−11% polyacrylamide gels, transferred to Immobilon-P membranes (Millipore) by electroblotting and probed using rabbit antiserum to *M. tuberculosis* KatG (from Dr C. Barry) or AhpC (Pagan-Ramos *et al*., 1998). Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Pierce) was used as the secondary antibody. Bound antibodies were visualized using ECL (NEN Research Products) as recommended. NIH IMAGE (version 1.62; National Institutes of Health) was used to quantify the relative intensity of KatG or AhpC proteins.

Enzyme activity stains

Catalase and peroxidase activities were assayed as described previously (Wayne and Diaz, 1986). Briefly, 50 μg of total unboiled protein extract were loaded on a 7.5% non-denaturing polyacrylamide gel and run at 100 V at 4°C. For catalase staining, polyacrylamide gels were soaked in 5 mM H_2O_2, briefly washed in water and incubated in a solution containing 2% ferric chloride and 2% potassium ferricyanide. Catalase activity was visible as a clear area on a green-stained background. For peroxidase staining, polyacrylamide gels were soaked in a solution of 0.5 mg ml^{-1} diaminobenzidine prepared in 5 mM H_2O_2. Peroxidase activity was visible as a brown band on an achromatic background. NIH IMAGE was used to quantify the relative intensity of catalase or peroxidase staining from polyacrylamide gels.

Statistical analysis

All statistical analyses (analysis of variance and Fisher's protected least significant difference) were performed with ANOVA (version 1.11; Abicus Software).

Nucleotide sequence accession numbers

The sequences reported here have been deposited in GenBank with the following accession numbers: (i) AF012631for *M. smegmatis furA*; (ii) AF092559 for *M. avium furA–katG* partial sequence; (iii) AF092558 for *M. aurum furA–katG* partial sequence; and (iv) AF092560 for *M. xenopi furA–katG* partial sequence.

Acknowledgements

We thank C. Barry for KatG antibodies, I. Smith for strain SM3, and E. Pagan-Ramos for useful comments. This work was supported by National Research Service Awards AI10278 to T.C.Z and AI10022 to J.S., and by grant AI42999 from the National Institute of Allergy and Infectious Disease to V.D.

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