Oxidative stress response genes in *Mycobacterium tuberculosis*: role of *ahpC* in resistance to peroxynitrite and stage-specific survival in macrophages

S. S. Master¹, B. Springer³, P. Sander³, E. C. Boettger⁴, V. Deretic¹ and G. S. Timmins²

Author for correspondence: V. Deretic. Tel: +1 505 272 0291. Fax: +1 505 272 6029.
e-mail: vderetic@salud.unm.edu

^{1,2} Department of Molecular Genetics and Microbiology¹ and Department of Pharmacy², University of New Mexico Health Sciences Center, 915 Camino de Salud, Albuquerque, NM 87131, USA

³ Institute for Medical Microbiology, Medizinische Hochschule, 30625, Hannover, Germany

⁴ Institute of Medical Microbiology, University of Zurich, CH-8028 Zurich, Switzerland

The *Mycobacterium tuberculosis ahpC* gene, encoding the mycobacterial orthologue of alkylhydroperoxide reductase, undergoes an unusual regulatory cycle. The levels of AhpC alternate between stages of expression silencing in virulent strains grown as aerated cultures, secondary to a natural loss of the regulatory *oxyR* function in all strains of the tubercle bacillus, and expression activation in static bacilli by a yet undefined mechanism. The reasons for this unorthodox regulatory cycle controlling expression of an antioxidant factor are currently not known. In this work, *M. tuberculosis* H37Rv and *Mycobacterium smegmatis* mc²155 *ahpC* knockout mutants were tested for sensitivity to reactive nitrogen intermediates, in particular peroxynitrite, a highly reactive combinatorial product of reactive nitrogen and oxygen species, and sensitivity to bactericidal mechanisms in resting and activated macrophages. Both *M. tuberculosis ahpC*::Km^r and *M. smegmatis ahpC*::Km^r showed increased susceptibility to peroxynitrite. In contrast, inactivation of *ahpC* in *M. tuberculosis* did not cause increased sensitivity to donors of NO alone. *M. tuberculosis ahpC*::Km^r also showed decreased survival in unstimulated macrophages, but the effect was no longer detectable upon IFN γ activation. These studies establish a specific role for *ahpC* in antioxidant defences involving peroxynitrite and most likely additional cidal mechanisms in macrophages, with the regulatory cycle likely contributing to survival upon coming out of the stationary phase during dormancy (latent infection) or upon transmission to a new host.

Keywords: *M. tuberculosis*, nitric oxide, *ahpC*, peroxynitrite, latency

INTRODUCTION

Peroxynitrite (ONOO⁻) and other reactive nitrogen and oxygen intermediates produced by macrophages play a role in host defence against invading bacteria (Akaki *et al.*, 2000; Darrah *et al.*, 2000; Karupiah *et al.*, 2000; Nathan & Shiloh, 2000; Paziak-Domanska *et al.*, 2000; Shiloh & Nathan, 2000; Yu *et al.*, 1999). Consequently, many bacterial pathogens have evolved protection mechanisms against reactive oxygen and nitrogen intermediates (Zahrt & Deretic, 2002). In myco-

bacteria, the *katG* and *ahpC* genes are the best studied factors from this class. The *katG* locus is genetically linked to the *furA* gene (Deretic *et al.*, 1997; Pagan-Ramos *et al.*, 1998), which encodes a homologue of the ferric uptake regulator (Baillon *et al.*, 1999; Bsai *et al.*, 1998; Dubrac & Touati, 2000; Hassett *et al.*, 1997; Lee *et al.*, 1998; Niederhoffer *et al.*, 1990; Tardat & Touati, 1993; van Vliet *et al.*, 1998, 1999; Zheng *et al.*, 1999). Interestingly, the locus encompassing *furA* and *katG* has been inactivated in *Mycobacterium leprae*, but has been preserved in all other mycobacteria (Cole, 1998; Deretic *et al.*, 1997; Nakata *et al.*, 1997; Pagan-Ramos *et al.*, 1998). Previous studies in our laboratory indicate that FurA negatively regulates KatG expression in mycobacteria (Zahrt *et al.*, 2001), and that there exists a dual and

Abbreviations: DETA nonoate, (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate).

stage-specific induction of the two *katG* promoters (Master *et al.*, 2001). The *katG* gene encodes a catalase-oxidase (Heym *et al.*, 1993), which *in vitro* has peroxynitritase and additional redox activities (Heym *et al.*, 1993; Magliozzo & Marcinkeviciene, 1997; Wengenack *et al.*, 1999) and has been shown to play a role in the virulence of *M. tuberculosis* (Cooper *et al.*, 2000; Li *et al.*, 1998; Manca *et al.*, 1999; Middlebrook & Kohn, 1953; Mitchison *et al.*, 1963; Morse *et al.*, 1954; Wilson *et al.*, 1995). The mycobacterial *ahpC* gene encodes an orthologue of bacterial alkyl hydroperoxidases (Chen *et al.*, 1998; Christman *et al.*, 1985; Cooper *et al.*, 2000; Dhandayuthapani *et al.*, 1996; Heym *et al.*, 1997; Jacobson *et al.*, 1989; Pagan-Ramos *et al.*, 1998; Sherman *et al.*, 1996; Springer *et al.*, 2001; Sreevatsan *et al.*, 1997; Wilson *et al.*, 1998). In most mycobacteria, the *ahpC* gene is linked to *oxyR* and is activated by its gene product, an orthologue of the global regulator OxyR, controlling the peroxide stress response in bacteria (Aslund *et al.*, 1999; Christman *et al.*, 1985; Storz & Altuvia, 1994). In *M. tuberculosis* as a species, and in all members of the *M. tuberculosis* complex, the *oxyR* gene is inactivated and represents a pseudogene (Deretic *et al.*, 1995, 1997). Recently, we have shown that there exists a second level of *ahpC* regulation independent of *oxyR* (Springer *et al.*, 2001). The *ahpC* gene is silenced in aerobic cultures of virulent *M. tuberculosis*, but is activated in statically grown organisms (Springer *et al.*, 2001). AhpC has been indirectly implicated in nitric oxide metabolism using expression in heterologous systems like *Salmonella* (Chen *et al.*, 1998). Purified AhpC has been shown to reduce hydroxyperoxide radicals (Chauhan & Mande, 2001; Hillas *et al.*, 2000) and has also been suggested to have peroxynitritase activity (Bryk *et al.*, 2000), but no direct analyses in mycobacteria have been carried out thus far.

Here, we continued our investigations of the role of *ahpC* in *M. tuberculosis* biology, specifically with respect to its proposed role in resistance to reactive nitrogen species and survival in macrophages. Using knockout strains of *ahpC* (*ahpC*::Km^r) in *M. tuberculosis* (Springer *et al.*, 2001) and *M. smegmatis* (Dhandayuthapani *et al.*, 1996), we compared the wild-type strains and their *ahpC* mutant derivatives for survival upon exposure to compounds producing reactive nitrogen intermediates and during infection of resting and activated macrophages.

METHODS

Bacterial strains. *M. smegmatis* mc²155 strains *ahpC*⁺ (wild-type), *ahpC*::Km^r (VD1865-6; Dhandayuthapani *et al.*, 1996) and *furA*::Km^r (JS106-1; Zahrt *et al.*, 2001), and *M. tuberculosis* H37Rv Str^r strains *ahpC*⁺ (RvTAM1424, wild-type) and *ahpC*::Km^r (RvTAM1424-1-1; Springer *et al.*, 2001) were constructed previously and the specificity of the mutations confirmed by genetic complementation (Dhandayuthapani *et al.*, 1996; Springer *et al.*, 2001; Zahrt *et al.*, 2001).

Media and growth conditions. The strains were grown until mid-exponential phase and/or stationary phase (as indicated)

on 7H9 (Difco) or 7H11 plates, supplemented with 0.5% Tween, 0.2% glycerol and OADC (oleic acid, 10% bovine serum fraction V, glucose and catalase). Bacteria were grown at 37 °C. All manipulations of live *M. tuberculosis* were carried out under Biosafety Level 3 conditions.

Chemicals. Both peroxynitrite and DETA nonoate {(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate)} were purchased from Alexis Corporation.

Detection of lipid peroxides. Lipid peroxides were detected using FOX II reagent, which provides a sensitive colorimetric assay for peroxides measured spectrophotometrically at 560 nm. FOX II reagent contains 90% methanol, 25 mM H₂SO₄, 250 µM ferrous sulfate heptahydrate (Sigma) and 100 µM xylene orange (Sigma) (Jiang *et al.*, 1992; Nourooz-Zadeh *et al.*, 1994; Wolff *et al.*, 1994). *M. smegmatis* mc²155 strains *ahpC*⁺ (wild-type), *ahpC*::Km^r and *furA*::Km^r were grown until mid-exponential phase. These cultures were then exposed to 1 mM peroxynitrite for five 3 min cycles at 37 °C. One-hundred microlitres of the treated culture was incubated for 10 min with 900 µl FOX II reagent to allow the reaction of peroxides. Experiments were carried out in triplicate and results quantified using a standard curve created with hydrogen peroxide.

Sensitivity assays and survival in macrophages. *M. tuberculosis* H37Rv *ahpC*⁺ and *ahpC*::Km^r were allowed to reach stationary phase. Similarly, *M. smegmatis* mc²155 strains *ahpC*⁺ (wild-type), *ahpC*::Km^r and *furA*::Km^r were grown until mid-exponential or stationary phase. These cultures were then exposed to various concentrations of peroxynitrite and DETA nonoate or used to infect J774A macrophages at an m.o.i. of 10:1 in the presence or absence of IFN γ (500 U ml⁻¹) and LPS (125 ng ml⁻¹). The results of treatment with these compounds and macrophage infections were assessed by plating and c.f.u. determination.

RESULTS AND DISCUSSION

Role of *ahpC* in mycobacterial survival against reactive nitrogen intermediates

M. smegmatis mc²155 strains *ahpC*⁺ (wild-type), *ahpC*::Km^r and *furA*::Km^r, and *M. tuberculosis* H37Rv Str^r strains *ahpC*⁺ (wild-type) and *ahpC*::Km^r were grown until mid-exponential phase and/or stationary phase as indicated. *M. smegmatis* mid-exponential-phase and stationary-phase cultures were exposed to peroxynitrite using repeated cycles of addition of fresh reagent to the culture (1, 2 and 5 cycles) of 3 min each at 37 °C (Fig. 1a). The half-life of peroxynitrite in neutral solution is measured in seconds so a 3 min exposure is sufficient to ensure its complete consumption. Our results showed increased sensitivity to peroxynitrite of the *ahpC*::Km^r mutant *M. smegmatis* strain compared to *ahpC*⁺ cells (Fig. 1a, b). In contrast to the *ahpC*::Km^r mutant, another *M. smegmatis* mutant (*furA*::Km^r) was as resistant to peroxynitrite as the *ahpC*⁺ (parental) strain. The differential sensitivity to peroxynitrite was observed irrespective of whether the strains were growing exponentially or had entered stationary phase (Fig. 1b).

Fig. 1. The *ahpC* gene is required for full resistance of *M. smegmatis* to peroxynitrite. (a) Survival of mid-exponential phase exponentially growing cultures of *M. smegmatis* mc²155 *ahpC*⁺ (■), *ahpC*::Km^r (●) and *furA*::Km^r (▲) upon exposure to 0.5 mM peroxynitrite for the indicated number of 3 min cycles. (b) Comparison of mid-exponential-phase (□) and stationary-phase (■) cultures of *M. smegmatis* mc²155 *ahpC*⁺, *ahpC*::Km^r and *furA*::Km^r exposed to 5 cycles of 1 mM peroxynitrite. Survival is expressed as percentage c.f.u. in untreated controls. **, *P* < 0.01 (ANOVA). Actual *P* values are given below points showing statistically significant differences.

Next, experiments were carried out using *M. tuberculosis* H37Rv. Since *ahpC* is not expressed in virulent *M. tuberculosis* grown with aeration (Springer *et al.*, 2001), but is expressed in statically grown cultures, *M. tuberculosis* H37Rv *ahpC*⁺ and its *ahpC*::Km^r derivative were grown without aeration as described previously (Springer *et al.*, 2001). Here too, after 5 cycles of peroxynitrite treatment, a significant increase in sensitivity to peroxynitrite was observed in *ahpC*::Km^r mutant cells (Fig. 2a). To test the sensitivity of *M. tuberculosis* H37Rv strains *ahpC*⁺ and *ahpC*::Km^r to NO alone, stationary-phase cultures were exposed to an NO donor, DETA nonoate. No detectable differences in survival were observed between the wild-type and the *ahpC*::Km^r mutant under the conditions tested (Fig. 2b), although DETA did have an overall inhibitory effect

Fig. 2. The *ahpC* gene is required for full resistance of *M. tuberculosis* H37Rv to peroxynitrite, but not to nitric oxide. (a) Survival of *M. tuberculosis* H37Rv *ahpC*⁺ and *ahpC*::Km^r upon exposure to 0.5 (■) and 1.0 mM (□) peroxynitrite. (b) Survival of *M. tuberculosis* H37Rv *ahpC*⁺ (■) and *ahpC*::Km^r (□) treated with the NO donor DETA nonoate. Stationary-phase cultures were exposed to the indicated concentrations for five 3 min cycles (peroxynitrite) or for 3 days (DETA) at 37 °C. Survival is expressed as percentage c.f.u. in untreated cultures. *, *P* = 0.05; **, *P* = 0.01 (ANOVA).

on *M. tuberculosis*, consistent with a mycobactericidal action of NO.

Elevated lipid peroxides in the absence of *ahpC*

Peroxyntirite can react to form several oxidizing species that react with lipids to form lipid peroxides (see Fig. 3a). To examine whether differences in lipid peroxidation could be detected among the three *M. smegmatis* mc²155 strains, *ahpC*⁺ (wild-type), *ahpC*::Km^r and *furA*::Km^r, cultures were treated with peroxynitrite as described in Methods and assayed spectrophotometrically using the FOX II reagent. Our results show that the *ahpC*::Km^r mutant strain produced the maximum amount of lipid peroxides (*P* < 0.5, ANOVA), while the *furA*::Km^r mutant, which constitutively expresses KatG (Zahrt *et al.*, 2001), produced the least amount of lipid peroxides (Fig. 3b). The correlation observed between these results and the sensitivity of the strains to

Fig. 3. Increased production of lipid peroxides in the absence of the *ahpC* gene. (a) Chemistry of the reaction of peroxynitrite-derived oxidizing species with lipids to form lipid peroxides. (b) Spectrophotometric detection of lipid hydroperoxides on exposure of *M. smegmatis* mc²155 *ahpC*⁺, *ahpC::Km*^r and *furA::Km*^r to 1 mM peroxynitrite for five cycles. Results are expressed as nmol peroxide produced ± SE, quantified using a standard curve generated with hydrogen peroxide.

Fig. 4. Decreased survival of *M. tuberculosis ahpC::Km*^r after 7 days infection in macrophages. Survival is expressed as percentage c.f.u. obtained with *ahpC*⁺ cells in unstimulated (■) J774A macrophages at day 7. I+L (□) macrophages were activated with 500 U IFN γ ml⁻¹ and 125 ng LPS ml⁻¹.

peroxynitrite (compare Fig. 1 with Fig. 3.) suggests that lipid peroxidation levels correlate with the killing of mycobacteria. These results indicate that AhpC protects mycobacteria from the deleterious effects of peroxynitrite-induced oxidation.

Role of AhpC in *M. tuberculosis* survival during macrophage infection

Statically grown cultures of *M. tuberculosis* H37Rv *ahpC*⁺ and *ahpC::Km*^r were allowed to reach stationary phase and then used to infect J774A macrophages at an m.o.i. of 10:1 in the presence or absence of IFN γ and LPS. No differences in survival were observed within the first 3 days of infection (data not shown). However, after 7 days of infection, the survival of the mutant and the wild-type differed by one order of magnitude in resting macrophages (Fig. 4, filled bars), indicating a contribution of *ahpC* to innate defences in unstimulated macrophages, although our data cannot exclude a role for AhpC under some untested, immune phase conditions. The difference between *ahpC*⁺ and *ahpC::Km*^r strains was abrogated in macrophages stimulated with IFN γ and LPS (Fig. 4, open bars). In conclusion, *ahpC* plays a role in *M. tuberculosis* survival in macrophages.

However, its action seems to be either independent of IFN γ -induced effectors (e.g. NO; compare results with NO donors in Fig. 2), or its contribution is masked by additional cidal mechanisms in activated macrophages or by activation of additional defence mechanisms in *M. tuberculosis*.

Conclusions

Our results indicate that an intact *ahpC* gene is preserved in *M. tuberculosis*, despite the loss of its activator *oxyR*, because it does confer a selective advantage under a subset of circumstances encountered by the organism during infection as modelled in this study. It is also likely that KatG and AhpC have partially overlapping defence activities, and that they undergo stage-specific and/or tissue-specific expression with compensatory activities, as previously noted (Dhandayuthapani *et al.*, 1996; Sherman *et al.*, 1996; Heym *et al.*, 1997; Master *et al.*, 2001; Musser, 1995; Wallis *et al.*, 1999, 2000). Thus, the absence of *oxyR* (Deretic *et al.*, 1995, 1997), the silencing of *ahpC* (Springer *et al.*, 2001), and its differential expression and infection-stage-specific induction (Springer *et al.*, 2001) most likely reflect adaptations of *M. tuberculosis* to various aspects of its infectious cycle. For example, upon transmission to a new host or possibly during initial stages of reactivation from latent infection, the probably stationary-phase *M. tuberculosis* infects naïve, resting monocytes where *ahpC* may play a role in resistance to the very early, innate cidal mechanisms in macrophages (as shown in Fig. 4.). Once IFN γ and other protective cytokines become available, *ahpC* may play a lesser role, as indicated by the loss of differential survival between *ahpC*⁺ and *ahpC::Km*^r cells in macrophages, although our studies do not permit us to rule out a role for AhpC under some other, untested conditions operating during the adaptive immunity stage of the host response to mycobacterial infection. We propose that, at the very minimum, *ahpC* plays a role of an early sentinel, as the tubercle bacillus comes out of the stationary phase during dormancy (latent infection) upon reactivation or upon transmission to a new host.

ACKNOWLEDGEMENTS

This work was supported by NIH grant AI42999.

REFERENCES

- Akaki, T., Tomioka, H., Shimizu, T., Dekio, S. & Sato, K. (2000). Comparative roles of free fatty acids with reactive nitrogen intermediates and reactive oxygen intermediates in expression of the anti-microbial activity of macrophages against *Mycobacterium tuberculosis*. *Clin Exp Immunol* **121**, 302–310.
- Aslund, F., Zheng, M., Beckwith, J. & Storz, G. (1999). Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc Natl Acad Sci USA* **96**, 6161–6165.
- Baillon, M. L., van Vliet, A. H., Ketley, J. M., Constantinidou, C. & Penn, C. W. (1999). An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen *Campylobacter jejuni*. *J Bacteriol* **181**, 4798–4804.
- Bryk, R., Griffin, P. & Nathan, C. (2000). Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* **407**, 211–215.
- Bsat, N., Herbig, A., Casillas-Martinez, L., Setlow, P. & 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.
- Chauhan, R. & Mande, S. C. (2001). Characterization of the *Mycobacterium tuberculosis* H37Rv alkyl hydroperoxidase AhpC points to the importance of ionic interactions in oligomerization and activity. *Biochem J* **354**, 209–215.
- Chen, L., Xie, Q. W. & Nathan, C. (1998). Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. *Mol Cell* **1**, 795–805.
- Christman, M. F., Morgan, R. W., Jacobson, F. S. & Ames, B. N. (1985). Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* **41**, 753–762.
- Cole, S. T. (1998). The *Mycobacterium leprae* genome project. *Int J Lepr Other Mycobact Dis* **66**, 589–591.
- Cooper, A. M., Segal, B. H., Frank, A. A., Holland, S. M. & Orme, I. M. (2000). Transient loss of resistance to pulmonary tuberculosis in p47^(phox-/-) mice. *Infect Immun* **68**, 1231–1234.
- Darrah, P. A., Hondalus, M. K., Chen, Q., Ischiropoulos, H. & Mosser, D. M. (2000). Cooperation between reactive oxygen and nitrogen intermediates in killing of *Rhodococcus equi* by activated macrophages. *Infect Immun* **68**, 3587–3593.
- Deretic, V., Philipp, W., Dhandayuthapani, S., Mudd, M. H., Curcic, R., Garbe, T., Heym, B., Via, L. E. & Cole, S. T. (1995). *Mycobacterium tuberculosis* is a natural mutant with an inactivated oxidative-stress regulatory gene: implications for sensitivity to isoniazid. *Mol Microbiol* **17**, 889–900.
- Deretic, V., Song, J. & Pagan-Ramos, E. (1997). Loss of *oxyR* in *Mycobacterium tuberculosis*. *Trends Microbiol* **5**, 367–372.
- Dhandayuthapani, S., Zhang, Y., Mudd, M. H. & Deretic, V. (1996). Oxidative stress response and its role in sensitivity to isonicotinic acid hydrazide in *Mycobacterium* species: characterization and inducibility of *ahpC* by peroxides in *M. smegmatis* and lack of expression in *M. aurum* and *M. tuberculosis*. *J Bacteriol* **178**, 3641–3649.
- Dubrac, S. & Touati, D. (2000). Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the *sodB* promoter. *J Bacteriol* **182**, 3802–3808.
- Hassett, D. J., Howell, M. L., Ochsner, U. A., Vasil, M. L., Johnson, Z. & Dean, G. E. (1997). An operon containing *fumC* and *sodA* encoding fumarase C and manganese superoxide dismutase is controlled by the ferric uptake regulator in *Pseudomonas aeruginosa*: *fur* mutants produce elevated alginate levels. *J Bacteriol* **179**, 1452–1459.
- Heym, B., Zhang, Y., Poulet, S., Young, D. & Cole, S. T. (1993). Characterization of the *katG* gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculosis*. *J Bacteriol* **175**, 4255–4259.
- Heym, B., Stavropoulos, E., Honore, N., Domenech, P., Saint-Joanis, B. T., Wilson, M., Collins, D. M., Colston, M. J. & Cole, S. T. (1997). Effects of overexpression of the alkyl hydroperoxide reductase AhpC on the virulence and isoniazid resistance of *Mycobacterium tuberculosis*. *Infect Immun* **65**, 1395–1401.
- Hillas, P. J., del Alba, F. S., Oyarzabal, J., Wilks, A. & Ortiz De Montellano, P. R. (2000). The AhpC and AhpD antioxidant defense system of *Mycobacterium tuberculosis*. *J Biol Chem* **275**, 18801–18809.
- Jacobson, F. S., Morgan, R. W., Christman, M. F. & Ames, B. N. (1989). An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. *J Biol Chem* **264**, 1488–1496.
- Jiang, Z.-Y., Hunt, J. V. & Wolff, S. P. (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low-density lipoprotein. *Ann Biochem* **202**, 384–389.
- Karupiah, G., Hunt, N. H., King, N. J. & Chaudhri, G. (2000). NADPH oxidase, Nramp1 and nitric oxide synthase 2 in the host antimicrobial response. *Rev Immunogenet* **2**, 387–415.
- Lee, H. S., Lee, Y. S., Kim, H. S., Choi, J. Y., Hassan, H. M. & Chung, M. H. (1998). Mechanism of regulation of 8-hydroxyguanine endonuclease by oxidative stress: roles of FNR, ArcA, and Fur. *Free Radic Biol Med* **24**, 1193–1201.
- Li, Z., Kelley, C., Collins, F., Rouse, D. & 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.
- Magliozzo, R. S. & Marcinkeviciene, J. A. (1997). The role of Mn(II)-peroxidase activity of mycobacterial catalase-peroxidase in activation of the antibiotic isoniazid. *J Biol Chem* **272**, 8867–8870.
- Manca, C., Paul, S., Barry, C. E., 3rd, Freedman, V. H. & Kaplan, G. (1999). *Mycobacterium tuberculosis* catalase and peroxidase activities and resistance to oxidative killing in human monocytes *in vitro*. *Infect Immun* **67**, 74–79.
- Master, S., Zahrt, T. C., Song, J. & Deretic, V. (2001). Mapping of *Mycobacterium tuberculosis katG* promoters and their differential expression in infected macrophages. *J Bacteriol* **183**, 4033–4039.
- Middlebrook, G. & Kohn, M. L. (1953). Some observations on the pathogenicity of isoniazid resistant variants of the tubercle bacilli. *Science* **118**, 297–299.
- Mitchison, D. A., Selkon, J. B. & Lloyd, J. (1963). Virulence in the guinea pig, susceptibility to hydrogen peroxide, and catalase activity of isoniazid-sensitive tubercle bacilli from South Indian and British patients. *J Pathol Bacteriol* **86**, 377–386.
- Morse, W. C., Weiser, O. L., Kuhns, D. M., Fusillo, M., Dail, M. C. & Evans, J. R. (1954). Study of the virulence of isoniazid-resistant tubercle bacilli in guinea pigs and mice. *Am Rev Tuberc* **69**, 464–468.
- Musser, J. M. (1995). Antimicrobial agent resistance in mycobacteria: molecular genetic insights. *Clin Microbiol Rev* **8**, 496–514.
- Nakata, N., Matsuoka, M., Kashiwabara, Y., Okada, N. & Sasakawa, C. (1997). Nucleotide sequence of the *Mycobacterium leprae katG* region. *J Bacteriol* **179**, 3053–3057.

- Nathan, C. & Shiloh, M. U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A* **97**, 8841–8848.
- Niederhoffer, E. C., Naranjo, C. M., Bradley, K. L. & Fee, J. A. (1990). Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus. *J Bacteriol* **172**, 1930–1938.
- Nourooz-Zadeh, J., Tajaddini-Sarmadi, J. & Wolff, S. P. (1994). Measurement of plasma hydroperoxide concentrations by the ferrous oxidation xylenol orange assay in conjunction with triphenylphosphine. *Anal Biochem* **220**, 403–409.
- Pagan-Ramos, E., Song, J., McFalone, M., Mudd, M. H. & Deretic, V. (1998). Oxidative stress response and characterization of the *oxyR-ahpC* and *furA-katG* loci in *Mycobacterium marinum*. *J Bacteriol* **180**, 4856–4864.
- Paziak-Domanska, B., Klink, M., Jurkiewicz, M. & Rudnicka, W. (2000). Production of reactive nitrogen and oxygen intermediates in human granulocytes and monocytes during internalization of live BCG bacilli. *Med Dosw Mikrobiol* **52**, 353–360.
- Sherman, D. R., Mdluli, K., Hickey, M. J., Arain, T. M., Morris, S. L., Barry, C. E. & Stover, C. K. (1996). Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science* **272**, 1641–1643.
- Shiloh, M. U. & Nathan, C. F. (2000). Reactive nitrogen intermediates and the pathogenesis of *Salmonella* and mycobacteria. *Curr Opin Microbiol* **3**, 35–42.
- Springer, B., Master, S., Sander, P. & 7 other authors (2001). Silencing the oxidative stress response in *Mycobacterium tuberculosis*: Expression patterns of *ahpC* in virulent and avirulent strains and the effect of *ahpC* inactivation. *Infect Immun* **69**, 5967–5973.
- Sreevatsan, S., Pan, X., Zhang, Y., Deretic, V. & 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.
- Storz, G. & Altuvia, S. (1994). OxyR regulon. *Methods Enzymol* **234**, 217–223.
- Tardat, B. & Touati, D. (1993). Iron and oxygen regulation of *Escherichia coli* MnSOD expression: competition between the global regulators Fur and ArcA for binding to DNA. *Mol Microbiol* **9**, 53–63.
- van Vliet, A. H., Wooldridge, K. G. & Ketley, J. M. (1998). Iron-responsive gene regulation in a *Campylobacter jejuni fur* mutant. *J Bacteriol* **180**, 5291–5298.
- van Vliet, A. H., Baillon, M. L., Penn, C. W. & 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.
- Wallis, R. S., Patil, S., Cheon, S. H. & 12 other authors (1999). Drug tolerance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **43**, 2600–2606.
- Wallis, R. S., Perkins, M. D., Phillips, M. & 10 other authors (2000). Predicting the outcome of therapy for pulmonary tuberculosis. *Am J Respir Crit Care Med* **161**, 1076–1080.
- Wengenack, N. L., Jensen, M. P., Rusnak, F. & Stern, M. K. (1999). *Mycobacterium tuberculosis* KatG is a peroxynitritase. *Biochem Biophys Res Commun* **256**, 485–487.
- Wilson, T., de Lisle, G. W., Marcinkeviciene, J. A., Blanchard, J. S. & Collins, D. M. (1998). Antisense RNA to *ahpC*, an oxidative stress defence gene involved in isoniazid resistance, indicates that AhpC of *Mycobacterium bovis* has virulence properties. *Microbiology* **144**, 2687–2695.
- Wilson, T. M., de Lisle, G. W. & Collins, D. M. (1995). Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium bovis*. *Mol Microbiol* **15**, 1009–1015.
- Wolff, S. P. (1994). Ferrous ion oxidation in the presence of ferric iron indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol* **223**, 182–189.
- Yu, K., Mitchell, C., Xing, Y., Magliozzo, R. S., Bloom, B. R. & Chan, J. (1999). Toxicity of nitrogen oxides and related oxidants on mycobacteria: *M. tuberculosis* is resistant to peroxynitrite anion. *Tuber Lung Dis* **79**, 191–198.
- Zahrt, T. C. & Deretic, V. (2002). Reactive nitrogen and oxygen intermediates and bacterial defenses: unusual adaptations in *Mycobacterium tuberculosis*. *Antioxid Redox Signal* **4**, 141–159.
- Zahrt, T. C., Song, J., Siple, J. & Deretic, V. (2001). Mycobacterial FurA is a negative regulator of catalase-peroxidase gene *katG*. *Mol Microbiol* **39**, 1174–1185.
- Zheng, M., Doan, B., Schneider, T. D. & Storz, G. (1999). OxyR and SoxRS regulation of *fur*. *J Bacteriol* **181**, 4639–4643.

Received 22 March 2002; revised 22 June 2002; accepted 26 July 2002.