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

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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* and *M. smegmatis* *ahpC*::*Km* 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* also showed decreased survival in unstimulated macrophages, but the effect was no longer detectable upon IFNγ 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 mycobacteria, 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; Bs at et al., 1998; Dubrac & Touati, 2000; Hassett et al., 1997; Lee et al., 1998; Niederhofer 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-ammonio-ethyl)amino]diazen-1-ium-1,2-dio late).
stage-specific induction of the two katG promoters (Master et al., 2001). The katG gene encodes a catalase-peroxidase (Heym et al., 1993), which in vivo has peroxynitritase and additional redox activities (Heym et al., 1993; Magliozzo & Marcinkeviciene, 1997; Wegenack 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 hydroperoxides (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 activated 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 growing 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') 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' (VD1865-6; Dhandayuthapani et al., 1996) and furA::Km' (JS106-1; Zahrt et al., 2001), and M. tuberculosis H37Rv Str² strains ahpC⁺ (RvTAM1424, wild-type) and ahpC::Km' (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\}diozen-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' and furA::Km' 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' were allowed to reach stationary phase. Similarly, M. smegmatis mc²155 strains ahpC⁺ (wild-type), ahpC::Km' and furA::Km' 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 IFNγ (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' and furA::Km', and M. tuberculosis H37Rv Str² strains ahpC⁺ (wild-type) and ahpC::Km' 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' mutant M. smegmatis strain compared to ahpC⁺ cells (Fig. 1a, b). In contrast to the ahpC::Km' mutant, another M. smegmatis mutant (furA::Km') 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).
M. tuberculosis AhpC and peroxynitrite

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\(^\text{r}\) 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\(^\text{r}\) mutant cells (Fig. 2a). To test the sensitivity of M. tuberculosis H37Rv strains ahpC⁺ and ahpC::Km\(^\text{r}\) 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\(^\text{r}\) mutant under the conditions tested (Fig. 2b), although DETA did have an overall inhibitory effect on M. tuberculosis, consistent with a mycobactericidal action of NO.

Elevated lipid peroxides in the absence of ahpC

Peroxynitrite 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\(^\text{r}\) and furA::Km\(^\text{r}\), cultures were treated with peroxynitrite as described in Methods and assayed spectrophotometrically using the FOX II reagent. Our results show that the ahpC::Km\(^\text{r}\) mutant strain produced the maximum amount of lipid peroxides (\(P < 0.5\), ANOVA), while the furA::Km\(^\text{r}\) 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

![Graph showing percentage survival vs. No. of cycles for M. smegmatis strains](image1)

![Graph showing percentage survival vs. DETA (mM) for M. smegmatis strains](image2)

**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\(^\text{r}\) (▲) and furA::Km\(^\text{r}\) (▲) 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\(^\text{r}\) and furA::Km\(^\text{r}\) exposed to 5 cycles of 1 mM peroxynitrite. Survival is expressed as percentage c.f.u. in untreated controls. ***, \(P < 0.001\) (ANOVA). Actual \(P\) values are given below points showing statistically significant differences.

**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\(^\text{r}\) upon exposure to 0.5 (■) and 10 mM (▲) peroxynitrite. (b) Survival of M. tuberculosis H37Rv ahpC⁺ (■) and ahpC::Km\(^\text{r}\) (▲) 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).
Role of AhpC in M. tuberculosis survival during macrophage infection

Statically grown cultures of M. tuberculosis H37Rv ahpC+ and ahpC::Km' 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 IFNγ 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' strains was abrogated in macrophages stimulated with IFNγ 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 IFNγ-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 IFNγ 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' 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.

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Fig. 4. Decreased survival of M. tuberculosis ahpC::Km' 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 IFNγ ml−1 and 125 ng LPS ml−1.

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' and furA::Km' to 1 mM peroxynitrite for five cycles. Results are expressed as nmol peroxide produced ± SE, quantified using a standard curve generated with hydrogen peroxide.
REFERENCES


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