Antisense RNA to *ahpC*, an oxidative stress defence gene involved in isoniazid resistance, indicates that AhpC of *Mycobacterium bovis* has virulence properties

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Antisense RNA is a versatile tool for reducing gene expression. It was used to determine if *ahpC*, a gene that is involved in defence against oxidative stress and isoniazid (INH) resistance, is important for virulence of *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex. Antisense RNA constructs of *ahpC* were made using different strength promoters in front of a reversed coding sequence of *ahpC*. These constructs were electroporated into a virulent wild-type *M. bovis* strain and a moderately virulent INH-resistant *M. bovis* strain that was catalase/peroxidase-negative. Down-regulation of protein synthesis occurred and this was visualized by immunoblotting. All strains containing antisense RNA were markedly less virulent than their parent strains in guinea pigs. *M. bovis* with an up-regulated *ahpC* gene was more resistant to cumene hydroperoxide than its parent strain, which had a wild-type *ahpC* promoter. These results agree with a model of INH resistance in which overexpression of AhpC compensates in some INH-resistant strains for loss of catalase/peroxidase by maintaining the ability to defend against oxidative stress mediated through organic peroxides. In addition, normal expression of AhpC is crucial for maintaining the virulence of wild-type *M. bovis*, which has normal catalase/peroxidase levels.

Keywords: antisense RNA, AhpC, virulence, *Mycobacterium tuberculosis* complex, oxidative stress

INTRODUCTION

The oxidative stress response of mycobacteria is an emerging research area that is linked to resistance to isoniazid (INH), one of the front-line drugs used in the treatment of human tuberculosis. INH-resistant strains of *Mycobacterium tuberculosis* appeared soon after INH treatment was initiated in 1952 and these had often lost virulence for guinea pigs and had a simultaneous loss of catalase activity (Middlebrook, 1954). Catalase detoxifies hydrogen peroxide which is generated by normal bacterial metabolism as well as by macrophages as part of the host's defence mechanisms. The *M. tuberculosis* complex, which includes *Mycobacterium bovis*, contains a single catalase enzyme which also has

peroxidase activity and is encoded by *katG*. Several lines of evidence support the theory that INH is a prodrug that requires activation by KatG (Johnsson & Schultz, 1994; Basso *et al.*, 1996). Once INH is activated it inhibits the biosynthesis of cell wall mycolic acids by directly inhibiting an enoyl-[acyl-carrier-protein] reductase encoded by *inhA* (Dessen *et al.*, 1995; Quemard *et al.*, 1995, 1996). Activated INH may also inhibit other enzymes of mycolic acid synthesis (Mdluli *et al.*, 1996). Mutations which appear to confer low-level INH resistance have been identified in the coding sequence of *inhA* (Banerjee *et al.*, 1994; Kapur *et al.*, 1995), and more commonly in the *inhA* promoter (Musser *et al.*, 1996; Rouse *et al.*, 1995). However, one of the major means by which *M. tuberculosis* and *M. bovis* develop resistance to INH is by loss of functional KatG (Zhang & Young, 1994; Heym *et al.*, 1995). This leaves the organism more susceptible to damage from

Abbreviations: INH, isoniazid; TEA, triethanolamine.

Table 1. Mycobacterial strains and integrating shuttle plasmids used in this study

Strain/plasmid	Characteristics	Source/reference*
Strains		
<i>M. bovis</i>		
ATCC 35723	Bovine isolate	ATCC
ATCC 35728	INH-resistant daughter strain of ATCC 35723	ATCC
ATCC 35729	INH-resistant daughter strain of ATCC 35723	ATCC
WAg201	New Zealand bovine isolate	Wilson <i>et al.</i> (1995)
WAg405	INH-resistant daughter strain of WAg201, catalase-minus, no mutation in <i>ahpC</i> promoter	Wilson <i>et al.</i> (1995)
WAg420	INH-resistant daughter strain of ATCC 35723 (isolated <i>in vitro</i> on 5 µg INH ml ⁻¹)	This work
WAg421	ATCC 35723 carrying <i>ahpC</i> antisense RNA construct, pUHA244	This work
WAg422	ATCC 35723 carrying <i>ahpC</i> antisense RNA construct, pUHA245	This work
WAg423	ATCC 35728 carrying <i>ahpC</i> antisense RNA construct, pUHA244	This work
WAg424	ATCC 35728 carrying <i>ahpC</i> antisense RNA construct, pUHA245	This work
WAg427	ATCC 35728 carrying <i>ahpC</i> antisense RNA and <i>katG</i> construct, pUHA253	This work
<i>M. tuberculosis</i>		
H37Rv	TMC 102	TMC
ATCC 35822	INH-resistant daughter of H37Rv	ATCC
Plasmids		
pYUB178	Integrating mycobacterial shuttle plasmid	Pascopeella <i>et al.</i> (1994)
pUHA244	pYUB178 carrying ATCC 35723 <i>ahpC</i> promoter in front of reversed coding sequence of <i>ahpC</i>	This work
pUHA245	pYUB178 carrying ATCC 35822 <i>ahpC</i> promoter in front of reversed coding sequence of <i>ahpC</i>	This work
pUHA253	pUHA245 carrying a <i>katG</i> gene integrated into the <i>KpnI</i> site	This work

* ATCC, American Type Culture Collection; TMC, Trudeau Mycobacterial Collection.

hydrogen peroxide and other toxic lipid peroxides. While this does not inhibit growth of the organisms in mycobacterial culture media, some catalase-minus INH-resistant strains are no longer virulent, probably because they require a functional KatG for protection against the oxidative burst of macrophages. Restoration of KatG activity to *M. tuberculosis* (Zhang *et al.*, 1993) or *M. bovis* (Wilson *et al.*, 1995) restores INH sensitivity and in the case of *M. bovis* has been shown to restore virulence for guinea pigs (Wilson *et al.*, 1995).

AhpC, a putative alkyl hydroperoxide reductase, appears to be produced constitutively in wild-type strains of the *M. tuberculosis* complex and its level does not respond to oxidative stress (Sherman *et al.*, 1995). In many bacteria, the response to oxidative stress is determined by a central regulator, OxyR, which controls the transcription of genes such as *katG* and *ahpC* (Christman *et al.*, 1985; Storz *et al.*, 1989). Although the *oxyR* gene is functional in *Mycobacterium leprae* and *Mycobacterium avium*, it is inactive in *M. tuberculosis* and *M. bovis* (Deretic *et al.*, 1995; Dhandayuthapani *et al.*, 1996; Sherman *et al.*, 1995). Insertional inactivation of *ahpC* in *Mycobacterium smegmatis* increased the susceptibility of this species to INH (Zhang *et al.*, 1996) and increased production of AhpC in *M. smegmatis* decreased its susceptibility (Dhandayuthapani *et al.*, 1996). This correlation with the level of INH sus-

ceptibility suggested that AhpC might have been detoxifying the active form of INH produced by KatG. However, a similar effect with AhpC has not been shown in strains of the *M. tuberculosis* complex.

Some INH-resistant strains of the *M. tuberculosis* complex have mutations in the promoter of *ahpC*. These mutations in the *ahpC* promoter lead to increased expression of AhpC as determined by reporter assays and immunoblotting (Wilson & Collins, 1996; Heym *et al.*, 1997; Sherman *et al.*, 1996). It appears that to survive attack from both the host and the presence of INH, there has been a selection for strains that have lost catalase/peroxidase activity and gained a compensating increase in expression of AhpC.

After unsuccessful attempts to inactivate the *ahpC* gene in *M. bovis* by allelic exchange, we developed an antisense RNA approach to reduce the levels of AhpC. Bacteria and phage use endogenous antisense RNA transcripts as an efficient and specific means of regulating gene expression of several biological functions (Wagner & Simons, 1994). Antisense RNA is increasingly being used as a research tool for reducing gene expression. It may be especially useful in situations where it is lethal to inactivate a gene but reduction of the level of protein might be tolerated. With the use of this approach, together with AhpC immunoblotting, we investigated the role of AhpC in a wild-type strain of *M.*

bovis with a normal *ahpC* promoter and in an INH-resistant strain of *M. bovis* with an up-regulated *ahpC* promoter.

METHODS

Mycobacterial strains, growth conditions and catalase activity. Mycobacterial strains and plasmids used in this study are given in Table 1. Strains of *M. bovis* were routinely cultured in Middlebrook 7H9 (Difco) liquid medium supplemented with albumin/glucose (Difco), 0.05% Tween 80 and 0.4% sodium pyruvate and in Middlebrook 7H11 (Difco) solid medium supplemented with albumin/glucose and 0.4% sodium pyruvate. Strains were electroporated by using the method of Wards & Collins (1996) in which slow-growing mycobacteria are washed at room temperature and electroporated at 37 °C. After electroporation, *M. bovis* was cultured on solid medium containing 15 µg kanamycin ml⁻¹. ATCC 35728 used in this study was originally isolated as an INH-resistant strain from a rabbit that was inoculated with ATCC 35723 and treated with INH (Steenken & Wolinsky, 1955). WAg420 was isolated by selecting colonies of ATCC 35723 for resistance to 5 µg INH ml⁻¹ on solid medium. It was subsequently determined to be catalase-negative and to have an MIC for INH of > 50 µg ml⁻¹ by using previously described methods (Wilson *et al.*, 1995).

Purification of AhpC. The coding sequence of *ahpC* was amplified using primers 5'-TTAATTCCATATGCCACTGCTAACCATTTGG and 5'-CTCGGATCCTTAGGCCGAAGCCTTGAGGA, digested with *Bam*HI and *Nde*I, directionally cloned into pET3a (Novagen), and electroporated into *Escherichia coli* BL21. A culture was grown overnight at 34 °C and expression of AhpC was induced by IPTG. After 2 h, the bacteria were pelleted and suspended in 50 mM triethanolamine/HCl buffer, pH 7.8 (TEA), containing protease inhibitors (2.3 mg leupeptin l⁻¹; 52 mg 1-chloro-3-tosylamido-7-amino-2-heptadone l⁻¹; 20 mg soybean trypsin inhibitor l⁻¹; 1.6 mg aprotinin l⁻¹; 1.1 mg pepstatin l⁻¹; 36.2 mg PMSF l⁻¹; and 1 mg lysozyme ml⁻¹). Cells were disrupted by sonication, the cell debris was pelleted and nucleic acids were precipitated by the addition of streptomycin sulphate to 1%. The supernatant was dialysed against TEA and loaded onto a Fast-Flow Q Sepharose (Pharmacia) column. Proteins were eluted using a 0–1 M NaCl gradient, and fractions containing the induced 25 kDa AhpC were pooled and loaded onto a Superdex 200 column. Eluted fractions containing the 25 kDa band were pooled and applied to a 1 × 10 cm Mono Q (Pharmacia) column. Proteins were eluted with a 0–0.5 M NaCl gradient and the desired fractions were pooled. The pool was applied to a Sephadex G-75 column and fractions containing the 25 kDa protein were pooled to yield 16 mg 95% pure protein. Antiserum against this purified AhpC was raised in female New Zealand White rabbits by intramuscular injection of 250 µg in Freund's incomplete adjuvant (Difco) followed by a subcutaneous booster of 250 µg after 3 weeks and a second booster after another 2 weeks. The rabbits were bled 2 weeks later and serum was collected.

Sensitivity growth curves. Sensitivity of *M. bovis* strains to inhibitory compounds was determined by adding 0.1 ml of a mid-exponential-phase culture in Tween-albumin broth (Kent & Kubica, 1985) to 5 ml amounts of the same broth which contained increasing concentrations of either cumene hydroperoxide (dissolved in DMSO) or hydrogen peroxide. The OD₆₀₀ was measured after 4 d (Ultrospec 2000 spectrophotometer; Pharmacia Biotechnology) and the percentage

Fig. 1. Schematic representation of the experimental procedure for making antisense RNA constructs. PW, *ahpC* promoter from ATCC 35723; P2, *ahpC* promoter from ATCC 35822; ♦, mutations in the *ahpC* promoter; ●, *Bam*HI sites; ■, *Bgl*II sites.

survival was calculated. A few samples were plated for c.f.u. to verify that OD₆₀₀ correlated to c.f.u.

Antisense constructs. The antisense constructs were generated as summarized in Fig. 1. The *ahpC* promoters used were a wild-type promoter (PW) from ATCC 35723 and a promoter with two mutations (P2) from ATCC 35822. PW and P2 were amplified separately with primers A (5'-CTCGGATCCACTGCTGAACCACTGCTTTGC) and B (5'-CTCGGATCCGACTCTCCTCATCATCAATGC), the *ahpC* coding sequence was amplified with primers C (5'-CTCGGATCCTCATGCCACTGCTAACCATTTG) and D (5'-CTCGGATCCTTAGGCCGAAGCCTTGAGGAG) and all PCR products were digested at the *Bam*HI sites that had been incorporated in the primers. PW and P2 were ligated separately to the coding sequence of *ahpC* and an aliquot of each ligation mix was subjected to PCR amplification with primers A2 (5'-GGAA-GATCTACTGCTGAACCACTGCTT) and C2 (5'-GGAAG-ATCTATGCCACTGCTAACCATTT), which bound at similar positions to primers A and C but incorporated *Bgl*II sites. The mixture was digested with *Bgl*II and ligated into the *Bcl*I cloning site of the integrating shuttle vector pYUB178. The ligation mixtures were electroporated into *E. coli* and kanamycin-resistant clones containing the expected 800 bp antisense inserts were selected. The two antisense constructs, pUHA244 for PW and pUHA245 for P2, were electroporated into ATCC 35723 and ATCC 35728. Kanamycin-resistant colonies were picked into liquid medium and after being cultured to mid-exponential phase their proteins were extracted (Wilson *et al.*, 1995). A third antisense construct was

made by ligating a 4.5 kb *KpnI* fragment containing *katG* into the *KpnI* site of pUHA245 to form pUHA253. This plasmid was electroporated into ATCC 35728.

Immunoblotting. Equal amounts of protein (determined by using a Bio-Rad kit with BSA as the protein standard) were separated on a 12% SDS-PAGE gel and transferred to nylon (Zeta-Probe; Bio-Rad) at 50 V for 45 min in 3 mM Na_2CO_3 , 10 mM NaHCO_3 , pH 9.9, containing 20% (v/v) methanol. The nylon was blocked with 5% skim milk powder in Tris/NaCl buffer (20 mM Tris, pH 7.5, 500 mM NaCl) for 1 h, incubated with anti-AhpC overnight at a 1:100 dilution, washed in Tris/NaCl buffer, incubated with goat anti-rabbit alkaline phosphatase conjugate (1:2000 dilution) for 4 h, washed again in Tris/NaCl buffer and developed using chloronaphthol solution (0.015 g chloronaphthol dissolved in 0.5 ml ethanol and added to 50 ml 50 mM Tris, pH 7.5, and 0.06% hydrogen peroxide).

Southern blotting. DNA was isolated from *M. bovis* strains (van Soolingen *et al.*, 1991) and digested with *PstI* overnight. DNA fragments were separated on a 1% agarose gel, transferred to nylon (Zetaprobe; Bio-Rad) and probed using ^{32}P -labelled DNA.

Determination of virulence for guinea pigs. The virulence of *M. bovis* strains was assessed in female Duncan-Hartley guinea pigs, using a method described previously (Wilson *et al.*, 1995). Each strain was inoculated at 10^6 c.f.u. into three guinea pigs which were housed isolated in a biohazard animal cage. All animals were skin tested with bovine purified protein derivative before inoculation and again before being killed 8 weeks later. The assessment of virulence was based on the presence of visible and microscopic lesions of tuberculosis and numbers of organisms present in the spleen. *M. bovis* was isolated from guinea pig spleens by homogenization of half the spleen with 20 ml water for 1 min. This was filtered through sterile cheesecloth and centrifuged at 3500 g for 20 min. The pellet was resuspended in 0.5 ml water and aliquots were plated onto Middlebrook 7H11 medium supplemented with 0.6 ml oleic acid l^{-1} , 50 g BSA l^{-1} , 20 g glucose l^{-1} , 7.7 g NaCl l^{-1} , 0.4% sodium pyruvate, 0.5% lysed sheep red blood cells, 10% bovine serum, 10 μg fungizone ml^{-1} , 200 IU polymyxin B sulphate, 100 μg ticarcillin ml^{-1} and 10 μg trimethoprim ml^{-1} . All animal work was approved by the institution's Animal Ethics Committee.

RESULTS AND DISCUSSION

Overexpression of AhpC

The coding sequence of *ahpC* was ligated into an expression vector and AhpC was expressed and purified by column chromatography. Antiserum was raised and probed against a Western blot of protein extracts from *M. tuberculosis* and *M. bovis* strains (Fig. 2). Strains with a wild-type *ahpC* promoter had barely detectable levels of AhpC. The promoters from the *ahpC* gene had previously been studied using a reporter assay (Wilson & Collins, 1996) and the immunoblot verified the trends previously identified. *M. tuberculosis* strain ATCC 35822, which has two mutations in the *ahpC* promoter, overexpressed AhpC by 20–30-fold, while *M. bovis* ATCC 35728 with one mutation overexpressed by 10–15-fold compared to their respective parent strains. Another INH-resistant *M. bovis* strain (WAg405) with no mutations in *ahpC* had no detectable increase in

Fig. 2. Immunoblot analysis of protein extracts (20 μg) from strains of *M. tuberculosis* and *M. bovis*, probed with polyclonal antiserum to AhpC. Parent strains are in bold, with their daughter strains shown to the right. S and R denote sensitivity or resistance to INH. All resistant strains had an MIC for INH of $>50 \mu\text{g ml}^{-1}$.

AhpC expression. Mutations in the promoter have been found in 13% of INH-resistant clinical strains of *M. tuberculosis* (Heym *et al.*, 1997; Telenti *et al.*, 1997), and mutations in the coding sequence have also been reported although their functional significance is unknown (Sreevatsan *et al.*, 1997). It was proposed by us and others that selection for up-regulation of *ahpC* occurred when an organism became INH-resistant through loss of KatG activity and that AhpC might be compensating for this loss (Wilson & Collins, 1996; Sherman *et al.*, 1996; Heym *et al.*, 1997).

Overexpression of AhpC was found to confer resistance to both hydrogen peroxide and the organic peroxide cumene hydroperoxide (Fig. 3). The parent ATCC 35723 (wild-type KatG and AhpC) was resistant to hydrogen peroxide but sensitive to cumene hydroperoxide; its *in vitro*-derived daughter, WAg420 (KatG-minus, AhpC wild-type), was very sensitive to hydrogen peroxide and sensitive to cumene hydroperoxide, while the *in vivo*-derived daughter, ATCC 35728 (KatG-minus, AhpC overexpressed), showed moderate resistance to both hydrogen peroxide and cumene hydroperoxide. This indicates that AhpC may play a role in protecting against oxidative stress.

Antisense RNA reduces production of AhpC

We attempted unsuccessfully to inactivate *ahpC* by allelic exchange with a fragment of DNA containing a drug-resistance marker interrupting the *ahpC* gene (data not shown) in a similar way to that used to interrupt the *ureC* gene (Reyrat *et al.*, 1995). This lack of success may have been either due to *ahpC* being an essential gene or may be a reflection of the difficulty in carrying out double homologous recombination in wild-type *M. bovis*. Based on the rationale that it might be lethal to inactivate *ahpC* but that reduction of the level of AhpC might be tolerated, we chose to reduce the level of AhpC by using antisense RNA.

An antisense RNA approach was designed so that the amount of free mRNA encoding AhpC would be reduced to two different extents resulting in reduced

Fig. 3. Growth sensitivity curves for *M. bovis* strains at different concentrations of hydrogen peroxide (a) and cumene hydroperoxide (b). ●, ATCC 35723; ■, ATCC 35728; ◆, WAg420.

levels of AhpC. Two antisense RNA constructs were made using a wild-type promoter from the INH-sensitive strain ATCC 35723 and a promoter from the INH-resistant strain ATCC 35822 that has two mutations which effect a large increase in AhpC levels (Fig. 2). These promoters were ligated in front of a reversed *ahpC* gene as shown diagrammatically in Fig. 1. The two constructs were electroporated into *M. bovis* strains ATCC 35723 and ATCC 35728. Integration of the constructs into the chromosome was confirmed by Southern blot analysis. The levels of AhpC in the recombinants and parent strains were determined by probing Western blots of protein extracts with anti-serum to AhpC (Fig. 4). The level of AhpC in ATCC 35728 was at least 10-fold higher than in its parent ATCC 35723, in which AhpC was barely detected. Reduction of the level of AhpC occurred in ATCC 35728 containing the antisense RNA with the wild-type promoter (WAg423) and the level of AhpC was lower still in the ATCC 35728 recombinant containing the antisense RNA with the up-regulated promoter (WAg424). No AhpC was detected on the Western blot with either of the antisense RNA constructs in ATCC 35723. Hence we could determine that the antisense RNA constructs had integrated into the chromosome and were reducing significantly the levels of AhpC.

Fig. 4. Immunoblot analysis of protein extracts (20 μg) from strains of *M. bovis*, probed with polyclonal antiserum to AhpC. Control extracts of the two strains ATCC 35728 and ATCC 35723 (lanes 1 and 4) are to the left of their respective recombinant daughter strains which contained the two different antisense RNA constructs. Lanes: 2, WAg423 containing the antisense construct with the wild-type promoter; 3, WAg424 containing the antisense construct with the promoter with two mutations; 5, WAg421 containing the antisense construct with the wild-type promoter; 6, WAg422 containing the antisense construct with the promoter with two mutations.

Antisense RNA constructs in *M. bovis* strains greatly reduced their virulence

The effect on virulence in guinea pigs of reducing the level of AhpC in *M. bovis* was assessed for the parent and antisense-containing strains on the basis of the presence or absence of gross and histopathological lesions in spleen, liver and lung tissues and on spleen c.f.u. (Table 2). The parent strain ATCC 35723 was virulent and multiple lesions were observed in the spleen and liver of all animals, while the antisense RNA recombinants (WAg421 and WAg422) caused no visible lesions and relatively few organisms could be cultured from the spleen. A reduction in virulence was also observed for the antisense recombinants of the INH-resistant ATCC 35728. ATCC 35728 itself was moderately virulent while the recombinant with the up-regulated promoter in front of the antisense RNA (WAg424) was assessed as avirulent.

Although immunoblotting showed that the levels of AhpC in WAg424 were similar to those in ATCC 35723, WAg424 was avirulent while ATCC 35723 was virulent. The only major difference known between these two strains was in their catalase/peroxidase activity encoded by *katG*. Thus we propose that, in moderately virulent strains such as ATCC 35728 which have no detectable KatG activity and which have gained an up-regulation mutation of their *ahpC* gene, the overexpression of AhpC partly compensates for the loss of virulence associated with loss of KatG activity.

While the above experiments clearly showed that incorporating antisense RNA to *ahpC* into *M. bovis* reduced the level of AhpC, the ability of this antisense RNA to reduce the virulence of *M. bovis* might not have been due to its effect on AhpC but to some other unforeseen effect such as titrating out an unknown regulatory factor. To rule out this possibility, an integrating plasmid was made which contained both an element producing antisense RNA to *ahpC* (with an up-

Table 2. Pathology of guinea pigs which had been subcutaneously inoculated with 10^6 c.f.u. and killed 8 weeks later

Strain	Guinea pig	Gross pathology*			Histopathology†			Culture‡
		Spleen	Liver	Lung	Spleen	Liver	Lung	
ATCC 35723	1	Yes	Yes	Yes	+++	++	+	20×10^3
	2	Yes	Yes	No	+++	++	—	9×10^3
	3	Yes	Yes	No	+++	++	—	12×10^3
WAg421	1	No	No	No	—	—	—	<20
	2	No	No	No	—	—	—	20
	3	Yes	No	No	+	—	—	30
WAg422	1	No	No	No	—	—	—	20
	2	Yes	No	No	+	—	—	300
	3	No	No	No	—	—	—	20
ATCC 35728	1	Yes	No	No	++	—	—	470
	2	Yes	No	No	++	—	—	530
	3	Yes	Yes	No	++	++	—	9×10^3
WAg423	1	No	No	No	+	—	—	320
	2	No	No	No	—	—	—	<20
	3	No	No	No	+	—	—	520
WAg424	1	No	No	No	—	—	—	<20
	2	No	No	No	—	—	—	<20
	3	No	No	No	—	—	—	<20
WAg427	1	Yes	Yes	No	+++	+++	—	44×10^3
	2	Yes	Yes	No	++	++	—	34×10^3
	3	Yes	Yes	Yes	+++	++	++	82×10^3

* Visible lesions observed.

† —, No evidence of lesions; +, ++, +++, degree of granuloma formation.

‡ c.f.u. values were corrected to represent the entire spleen.

regulated *ahpC* promoter) and a functional *katG* gene (pUHA253). This construct was electroporated into ATCC 35728 to produce a strain (WAg427) that was *katG*-positive (as shown by the production of bubbles when hydrogen peroxide was dropped onto a colony) and, because of the antisense element, would be expected to have a level of AhpC similar to that in WAg424. WAg427 was found to be virulent for guinea pigs (Table 2). So we had taken ATCC 35728 that was moderately virulent, reduced its virulence by reducing the expression of AhpC, and then been able to restore the organism to full virulence by restoring KatG activity. We conclude that the reduction in virulence due to the antisense RNA is a result of its action in reducing the level of AhpC and not because it has some other non-specific effect. It also appears from this experiment that the moderate virulence of ATCC 35728 is because of its lack of a functional *katG* gene.

AhpC also appears to perform a detoxification function that is independent of KatG. ATCC 35723, a virulent strain of *M. bovis* with wild-type levels of both KatG and AhpC, lost virulence in the presence of both levels of antisense RNA, indicating that the organism could not remain virulent with less than normal constitutive expression of AhpC. It should be noted that loss of

virulence and a reduced level of AhpC were the only phenotypic changes observed in the antisense-containing recombinants of ATCC 35723 and these recombinants had similar *in vitro* growth to their parent strain.

Antisense RNA revertants isolated from guinea pigs

M. bovis colonies isolated from guinea pig spleens after the 8 week virulence experiment were analysed by Southern blotting and hybridization with a probe of the vector pYUB178 (Fig. 5). This analysis revealed that DNA of colonies from some animals (Fig. 5a, lanes 2, 3, 6, 7 and 9) no longer hybridized to the vector that had been integrated even though there was an adequate amount of DNA in each of these lanes (Fig. 5b). In the results shown in Fig. 5 for four animals inoculated with either WAg421 or WAg423, three of the animals contained these revertants. We also saw this evidence of reversion in animals inoculated with the other antisense-containing *M. bovis* recombinants WAg422 and WAg424 (data not shown). All strains that were used for inoculation into guinea pigs came from single colony isolates that contained an integrated pYUB178 construct. Previously, we have made integrated pYUB178 recombinants with several different constructs and have never observed reversion of the strains under *in vitro* or

Fig. 5. Southern blot data for DNA from strains isolated from guinea pig (GP) spleens at the end of the virulence experiment. (a) Southern blot hybridization after probing with ^{32}P -labelled integration vector (pYUB178). (b) Photograph of the gel before transferring to nylon.

in vivo conditions (Collins *et al.*, 1995; Wilson *et al.*, 1995). These results indicate that there was a selective pressure in animals for these strains to lose the antisense constructs and regain their natural virulence. However, no evidence of reversion was found in any of the 12 samples of DNA from colonies isolated from three different animals inoculated with WAg427 (antisense plus *katG*) (Fig. 5). In this case, the antisense RNA has reduced the levels of AhpC from high to wild-type levels and this in the presence of a functional *katG* enabled WAg427 to be virulent. It therefore appears that in the presence of wild-type levels of activity of KatG and AhpC there was no selective advantage for the antisense construct to be lost.

In an alternative approach to investigate the role of AhpC in virulence, INH-resistant strains of *M. tuberculosis* were produced *in vitro* and it was found that a strain that had lost KatG activity and acquired an up-regulated *ahpC* was attenuated in mice (Heym *et al.*, 1997). Having an up-regulated *ahpC* in that strain did not increase virulence. While the results of this mouse experiment contrast with our present study this may be explained by a number of factors, including the different overall genetic composition of the strains used in the two studies, the different animal models used and the different ways in which strains were derived. The major difference between INH-resistant strains derived *in vitro* and those isolated clinically is their mode of selection. If the strain is selected in a host that is being treated with INH, there is a dual selective pressure to develop INH

resistance and to cope with the host defence mechanisms, including the oxidative challenge of the macrophage. It is possible that in these clinical strains derived *in vivo*, another as yet unknown genetic change may be compensating for the loss of that part of the KatG function that an overexpressed AhpC cannot fully replace.

This study has shown the utility of antisense RNA technology in investigation of mycobacterial virulence. The recent expression of antisense RNA to alpha toxin to attenuate *Staphylococcus aureus* confirms the general usefulness of such approaches for studying bacterial virulence (Kernodle *et al.*, 1997). The wider versatility of antisense RNA methods for mycobacterial studies has been demonstrated by the creation of histidine auxotrophs of *M. smegmatis* (Parish & Stoker, 1997) and by the use of antisense oligodeoxynucleotide phosphorothioates as antimycobacterial agents against drug-resistant *M. smegmatis* (Rapaport *et al.*, 1996). The main finding of this study is that AhpC plays an important role in the virulence of both wild-type and INH-resistant strains of *M. bovis* and that overexpression of AhpC can compensate for the loss of catalase/peroxidase activity that occurs in some INH-resistant strains.

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