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5′-Adenosinephosphosulphate reductase (CysH) protects *Mycobacterium tuberculosis* against free radicals during chronic infection phase in mice

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Summary

A major obstacle to tuberculosis (TB) control is the problem of chronic TB infection (CTBI). Here we report that 5′-adenosinephosphosulphate reductase (CysH), an enzyme essential for the production of reduced-sulphur-containing metabolites, is critical for *Mycobacterium tuberculosis* (Mtb) survival in chronic infection phase in mice. Disruption of *cysH* rendered Mtb auxotrophic for cysteine and methionine, and attenuated virulence in BALB/c and C57BL/6 immunocompetent mice. The mutant and wild-type Mtb replicated similarly during the acute phase of infection, but the mutant showed reduced viability during the persistent phase of the infection. The *cysH* mutant caused disease and death after 4–7 weeks of infection in four different groups of mice – Rag1$^{-/-}$, NOS2$^{-/-}$, gp91phox$^{-/-}$ NOS2$^{-/-}$ and gp91phox$^{-/-}$ mice given aminoguanidine [to suppress the effects of nitric oxide synthase 2 (NOS2)] – indicating minimal metabolic effect on the *cysH* mutant survival in these mice. The *cysH* mutant was also susceptible to peroxynitrite and hydrogen peroxide *in vitro*. These results show that CysH is important for Mtb protection during the chronic infection phase, and that resistance to nitrosative and oxidative stress may be the mechanism of this protection. Thus, this metabolic gene of an intracellular pathogen could have a secondary role in protection against the host immune response. Finally the lack of an endogenous human orthologue of *cysH* and its possible role in defence against adaptive immunity renders CysH an attractive enzyme for further studies as a target for therapeutics active against CTBI.

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Introduction

Each year, *Mycobacterium tuberculosis* (Mtb) causes an estimated 8 million cases of tuberculosis (TB) worldwide (Dye *et al.*, 2002). As one-third of the world's population is estimated to be chronically infected with Mtb, the control of this infection by the adaptive immune response is an area of considerable interest. AIDS patients are highly prone to reactivation from chronic TB infection (CTBI) (Williams and Dye, 2003; Chan and Flynn, 2004), indicating an important role for adaptive immunity in controlling TB.

In mice, T cells produce IFN-γ, which activates macrophages that destroy intracellular bacilli (Flynn and Chan, 2001). IFN-γ induces the expression of Ca^{++}-independent nitric oxide synthase 2 (NOS2), which is responsible for the production of reactive nitrogen intermediates (RNI) by macrophages (MacMicking *et al.*, 1997a; Ehrt *et al.*, 2001; Flynn and Chan, 2001). NOS2 also plays a major role in controlling Mtb persistence in mice; inhibition of NOS2 during the persistent phase of infection leads to reactivation of disease (Flynn and Chan, 2001) and mice lacking NOS2 cannot control Mtb infection (MacMicking *et al.*, 1997b).

The relevance of NOS2 in controlling Mtb infection in humans is less clear. Humans with TB have been shown to express enzymatically active NOS2, which is present in the inflammatory zone of granulomas in the lung; they exhale more nitric oxide than healthy controls (Nathan, 2002). Therefore it is possible, as in the mouse, that NOS2 plays a role in controlling Mtb infection in humans. A recent report shows that mouse NADPH oxidase [responsible for the production of reactive oxygen intermediates (ROI)] is also active in the persistent phase of the Mtb infection in mice (Ng *et al.*, 2004). However, despite these

reactive oxygen and nitrogen intermediate responses, Mtb is able to establish CTBI and must therefore have developed effective strategies to defend against oxidative and nitrosative stresses.

Reduced-sulphur-containing biomolecules are widely involved in antioxidant defence (Haramaki *et al*., 1997; Rawat *et al*., 2002; Buchmeier *et al*., 2003; Kwon *et al*., 2003) and we hypothesized that the pathway used for the production of reduced sulphur, the sulphur assimilation pathway, may be important for Mtb's survival. Indeed, sulphur assimilation genes are among those induced in mycobacteria when they take up residence within macrophages and when exposed to oxidative stress (Schnappinger *et al*., 2003; Pinto *et al*., 2004).

The first committed step in the sulphur assimilation pathway, conversion of adenosine 5′-phosphosulphate (APS) to sulphite (SO_3^{2-}), is catalysed by the enzyme CysH (Williams *et al*., 2002) (see Fig. S1). At the later stages, sulphite is further reduced to sulphide, and this fully reduced-sulphur metabolite is used in the biosynthesis of cysteine, methionine and a variety of cofactors. We recently demonstrated that disruption of *cysH* in *Mycobacterium smegmatis* confers cysteine and methionine auxotrophy (Williams *et al*., 2002). We also defined the catalytic mechanism of CysH (Carroll *et al*., 2005a,b). Here we report on the role of *cysH* in Mtb virulence and pathogenesis in mice.

Results

cysH-*deleted Mtb H37Rv is auxotrophic for methionine and cysteine*

A *cysH* deletion mutant (Δ*cysH*) was constructed in Mtb H37Rv. This deletion was confirmed by Southern hybridization (see Fig. S2). The mutant was complemented by expression of Mtb *cysH* from an extra-chromosomal (multicopy) plasmid (pMSGS:*cysH*) or an integrating vector (pMVGS:*cysH*). As expected, both complemented mutant strains were prototrophic for cysteine and methionine, whereas Δ*cysH* could not propagate unless the media were supplemented with either amino acid (see Fig. S3).

ΔcysH *is attenuated in mice*

The contribution of *cysH* to disease was assessed in mice. BALB/c mice were administered intravenously with either wild-type Mtb H37Rv (WT) or Δ*cysH*. Mice infected with WT succumbed within 21 weeks post infection (pi) with median survival (MS) of 19.5 weeks (Fig. 1A). All mice infected with Δ*cysH* continued to survive until the experiment was terminated at 45 weeks pi. The experiment was repeated with the addition of the complemented Δ*cysH* (pMSGS:*cysH*) strain. Both groups of mice infected with either WT or complemented Δ*cysH* succumbed within 29 weeks pi, with MS of 21 and 24 weeks respectively, while less than half of the mice in the Δ*cysH*-infected group succumbed to the infection before the experiment was terminated at 62 weeks pi (Fig. 1B).

CysH is indispensable for Mtb during the persistence phase of the infection

In the above experiment, growth kinetics of Δ*cysH*, WT and complemented Δ*cysH* (pMSG:*cysH*) were assessed in mouse lungs (Fig. 1C). Colony-forming units (cfu) of Δ*cysH* recovered from lungs were similar to that of WT in the acute phase of infection (until 16 days pi). In contrast, the number of Δ*cysH* bacilli recovered 42 days pi was significantly less than those of the two control strains. This reduction of bacilli between 16 and 42 days pi, when adaptive T_H1-mediated immunity develops and when Mtb enters persistence in mouse lung (Shi *et al*., 2003), compelled further study.

We performed additional Mtb infection studies with C57BL/6 mice, which are known to mount a stronger T_H1 type response than BALB/c against the Mtb complex strain BCG (Orme and Collins, 1994). For these experiments, we employed the more physiologically relevant inhalation route of infection. As observed with BALB/c mice, the cfu for Δ*cysH* were lower than WT and complemented Δ*cysH* in the persistent phase of the infection, which is characteristically demonstrated by a plateau in the growth curve (between 19 and 42 days pi, Fig. 1D). A higher number of cfu were recovered for complemented Δ*cysH* (pMSG:*cysH*) compared with WT in this experiment. This difference may be due to the multiple copies of *cysH* in complemented strain aiding the survival of bacteria in the more resistant C57BL/6 mouse strain.

Evidence for the attenuation of Δ*cysH* in C57BL/6 mice was further provided by lung pathology at different time points during the infection (Figs 2 and 5 and see Table S1). The replication of Δ*cysH* in mouse tissues during the first 19 days pi was unimpaired and identical to that of WT, suggesting that mouse tissues can provide Mtb with ample amounts of reduced-sulphur-containing substrates for growth. Therefore, CysH appears to be dispensable during the acute phase of infection. In contrast, CysH is indispensable in the later, persistence phase of infection.

CysH protects Mtb during persistent phase of infection

We considered two possible mechanisms by which the disruption of *cysH* might lead to attenuation of Mtb during the persistent phase of infection: (i) exhaustion of the required reduced-sulphur-containing substrates in mouse

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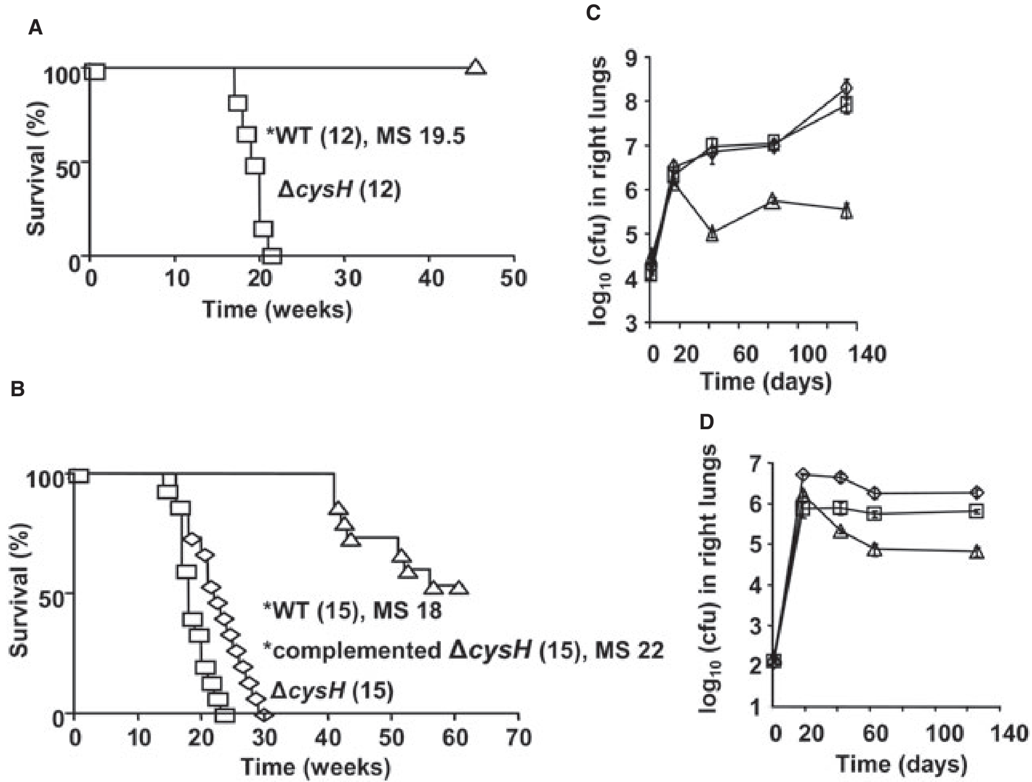

Fig. 1. Δ*cysH* is attenuated in BALB/c and C57BL/6 mice. Symbols for strains: WT (□), Δ*cysH* (△), complemented Δ*cysH* (pMGS:*cysH*) (◇).
A. Survival of BALB/c mice (*n* in brackets and MS is median survival) after intravenous infection with 10^6 cfu with Δ*cysH* or WT.
B. Survival of BALB/c mice after intravenous infection with 10^6 cfu with Δ*cysH*, WT or complemented Δ*cysH* (pMGS:*cysH*).
C. Right lung burdens for Δ*cysH*, WT or complemented Δ*cysH*-infected BALB/c mice, infected as in (A), $n = 3$ mice per group per time point.
D. Right lung bacterial burden for Δ*cysH*, WT or complemented Δ*cysH*-infected C57BL/6 mice after aerosol infection (right lungs were seeded with 100–200 bacilli), $n = 3$ mice per group per time point. Asterisk indicates $P < 0.0001$ as compared with Δ*cysH*-infected mice.

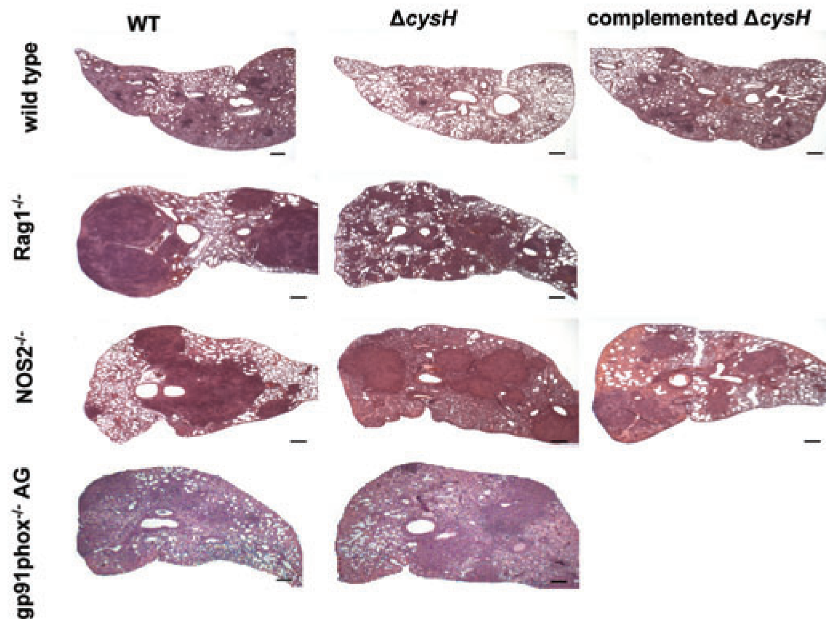

Fig. 2. Attenuation and virulence of Δ*cysH* by lung pathology. Magnified ×20 view of sections of lungs stained with H&E, scale bar, 300 μm. All the lungs from wild-type (C57BL/6) mice were harvested at 18 weeks pi while lungs from Rag1$^{-/-}$, NOS2$^{-/-}$ and gp91phox$^{-/-}$AG mice were harvested from dying mice. All the mice were infected by seeding lungs with aerosolized bacilli (100–200 bacilli per right lung). Above photographs were taken by a veterinary pathologist so as to represent each group at the given time point.

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lungs after the initial 19 days pi or (ii) CysH protects Mtb during the adaptive immune response phase.

To address the latter possibility, we infected Rag1$^{-/-}$ mice (in the C57BL/6 background), which lack functional T and B cells, with Δ*cysH* or WT. We also infected the mice with the vaccine strain *Mycobacterium bovis* strain BCG-P, to test the ability of Rag1$^{-/-}$ mice to control a known attenuated Mtb complex strain. All of the mice infected with WT succumbed within 31 days pi, while all of the Δ*cysH*-infected Rag1$^{-/-}$ mice succumbed to the infection within 55 days. The BCG-P-infected mice did not succumb until 263 days pi (Fig. 3A). These results suggest that Δ*cysH* is not limited by depletion of reduced-sulphur-containing metabolites that must be scavenged from mouse lungs: the bacteria replicated almost as well as WT throughout the course of infection, and were almost as virulent in Rag1$^{-/-}$ mouse (Fig. 3).

We analysed the lung pathology of Rag1$^{-/-}$ and C57BL/6 mice infected with Δ*cysH* (Fig. 2). Compared with the observation made in C57BL/6 mice, the rate of progression of disease in Rag1$^{-/-}$ mouse infected with the Δ*cysH* was faster, the extent and severity of the necrosis significantly more pronounced, and the numbers of acid fast bacilli were significantly higher (see Table S1 and Fig. S4). Together, these results demonstrate that CysH plays an important role in Mtb survival after adaptive immunity is established.

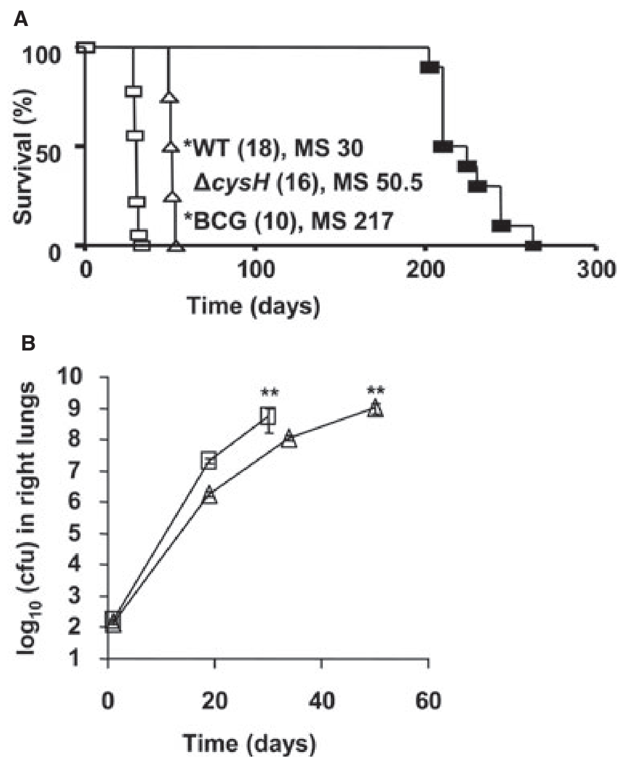

Fig. 3. Δ*cysH* is virulent in Rag1$^{-/-}$ mice.
A. Survival of Rag1$^{-/-}$ mice (*n* in brackets and MS is median survival) after aerosol infection (100–200 bacilli per right lung) with WT (□), Δ*cysH* (△) or BCG (■).
B. Right lung cfu for Δ*cysH* (△) or WT (□) infected Rag1$^{-/-}$ mice infected as in (A), $n = 3$ mice per group at day 1 and $n = 4$ per group at other time points. Double asterisks indicate dying mice.

CysH protects Mtb against the effects of NOS2

In mice, inducible NOS2 is known to play a vital role in controlling Mtb persistent infection (Flynn and Chan, 2001). In order to test the role of CysH in protecting the bacterium against the effects of NOS2, we infected NOS2$^{-/-}$ mice (C57BL/6 background) by the aerosol route with WT, Δ*cysH* and the two complemented Δ*cysH* strains. In contrast to the observation made in wild-type mice, Δ*cysH* did not lose viability after the first 21 days pi in NOS2$^{-/-}$ mice (Fig. 4A). We further confirmed this finding by infecting the NOS2$^{-/-}$ mice with Δ*cysH* at a lower inoculum dose (25 bacilli per right lung). Even with the lower inoculum dose, Δ*cysH* continued to replicate beyond the first 21 days of infection (Fig. 4A). WT and complemented Δ*cysH* strains were virulent in NOS2$^{-/-}$ mice; all mice succumbed to infection within 26–31 days (Fig. 4B). Compared with WT, Δ*cysH* replication slowed significantly after the first 21 days of infection (Fig. 4A). Nevertheless, Δ*cysH* continued to replicate in NOS2$^{-/-}$ mice and caused death in a relatively short period of time; the MS time was 82 days for a group of 12 mice (Fig. 4B). When similar inocula were used to infect the wild-type mice (C57BL/6: cognate strain of the NOS2$^{-/-}$ mouse) with WT, the MS time was 273 days for a group of 11 mice (data not shown). These results show that Δ*cysH* is significantly more virulent when NOS2 is absent in the mouse; indeed Δ*cysH* is more virulent in NOS2$^{-/-}$ mice than WT in wild-type mice (C57BL/6). The above results were further corroborated by lung pathology analysis of NOS2$^{-/-}$ and C57BL/6 mice infected with WT or Δ*cysH* (Figs 2 and 5 and see Table S1 and Fig. S4). Therefore CysH also appears to protect the bacteria against the effects of RNI induced by NOS2.

CysH protects Mtb against RNI and ROI in vitro

The results from the above *in vivo* studies were further corroborated by *in vitro* analysis of the sensitivity of Δ*cysH* and its two control strains to RNI and ROI. Recent reports suggest that peroxynitrite ($ONOO^-$), the product of the reaction of superoxide (O_2^-) and NO, may play an important role in controlling Mtb infection in the host (Yu *et al.*, 1999; Bryk *et al.*, 2000; Choi *et al.*, 2002). Therefore, we exposed the three strains to $ONOO^-$ to test their abilities to resist this bactericidal molecule. The viability of the bacteria after exposure to $ONOO^-$ was measured by quantifying cfu on plates. Δ*cysH* was significantly more sensitive to $ONOO^-$ than the two control strains (Fig. 6A and

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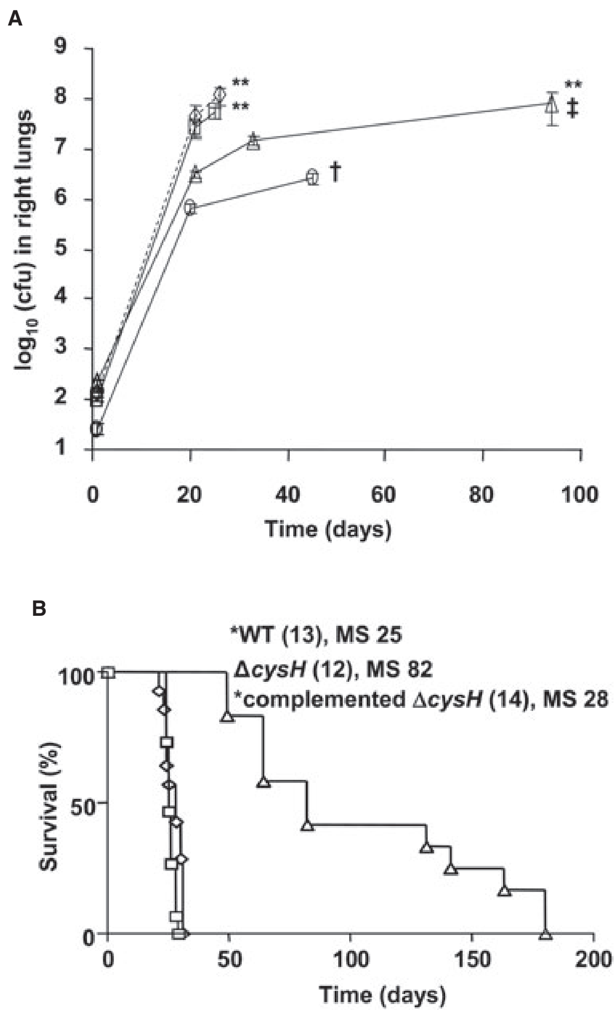

Fig. 4. Δ*cysH* is virulent in NOS2$^{-/-}$ mice.
A. Right lung burdens for Δ*cysH* (higher dose infection: △; lower dose infection: ○), WT (□), or complemented Δ*cysH* carrying the plasmid pMVGS:*cysH* (◇ and dash line) infected NOS2$^{-/-}$ mice after aerosol infection (100–200 bacilli per right lung, or 25 bacilli in experiment indicated by †), $n = 3$ mice per group at day 1, $n = 8$ at dying mice time point for Δ*cysH*-infected mice and $n = 4$ per group at other time points. ‡ = right lung burdens of eight mice infected with Δ*cysH*, dying at different pi times are shown at MS time of Δ*cysH*-infected NOS2$^{-/-}$ mice. Double asterisks indicate dying mice.
B. Survival of NOS2$^{-/-}$ mice after aerosol infection (100–200 bacilli per right lung) with either Δ*cysH* (△), WT (□) or complemented Δ*cysH* (◇). Complemented Δ*cysH*-infected mice are infected with either strain carrying the pMGS:*cysH* (six mice) or the strain carrying pMVGS:*cysH* (eight mice). Asterisk indicates $P < 0.0001$ as compared with Δ*cysH*-infected mice.

see Fig. S5). We also exposed the three strains to H_2O_2. This oxidant is derived from superoxide, which, in turn, is produced by NADPH oxidase in macrophages to control intracellular pathogens. As with $ONOO^-$, we consistently found Δ*cysH* to be more sensitive to H_2O_2 than the two control strains (Fig. 6B). This sensitivity of Δ*cysH* to H_2O_2 was interesting as H_2O_2 levels increase in response to INF-γ and Mtb infection in mouse macrophages (Nathan *et al*., 1983; Ehrt *et al*., 2001). The above experiments were performed after Δ*cysH* was incubated for 24 h without an exogenous supply of reduced sulphur in the medium. This incubation was performed to deplete the intracellular pool of reduced sulphur in Δ*cysH*. In the absence of such sulphur starvation, survival of Δ*cysH* after exposure to H_2O_2 or $ONOO^-$ was markedly increased: Δ*cysH* cfu recovered were only one half log less than those of WT (data not shown). However it must be noted that the above-mentioned starvation of reduced sulphur did not affect the survival of Δ*cysH* (when not exposed to H_2O_2 or $ONOO^-$) in the above experiments (Fig. 6 and see Fig. S5). Therefore, these experiments further show that once the bacteria have lower levels of reduced sulphur, they become more susceptible to radicals.

CysH protects Mtb against the effects of NADPH oxidase

To assess the relationship of the sensitivity of Δ*cysH* to H_2O_2 and the observed attenuation of Δ*cysH* (compared

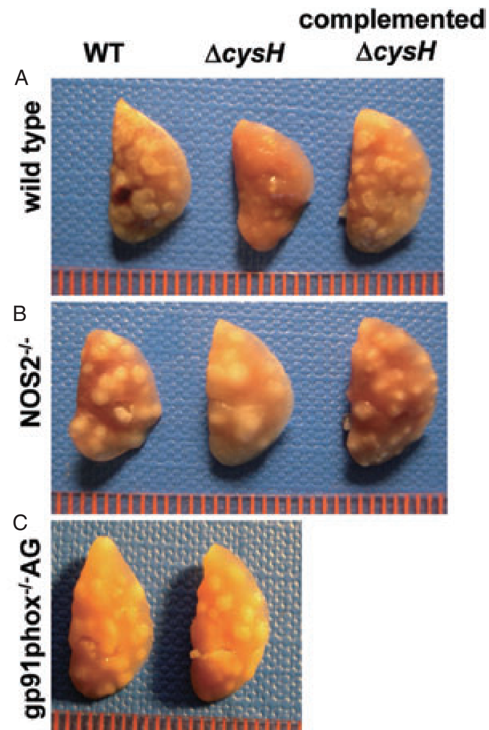

Fig. 5. Δ*cysH* is virulent in NOS2$^{-/-}$ and gp91phox$^{-/-}$AG mice by gross lung pathology.
A. Left lung pathology of wild-type (C57BL/6) mice infected as in Fig. 1D and harvested 18 weeks pi.
B. Left lung pathology of NOS2$^{-/-}$ mice infected as in Fig. 4B (100–200 bacilli per right lung). WT, complemented Δ*cysH* (pMGS:*cysH*) and Δ*cysH*-infected lungs are harvested at 27, 30 and 33 days pi respectively.
C. Left lung pathology of dying gp91phox$^{-/-}$AG mice after aerosol infection with WT or Δ*cysH* (approximately 100 bacilli per right lung). Scale in (A) (B) and (C) is 1 mm.

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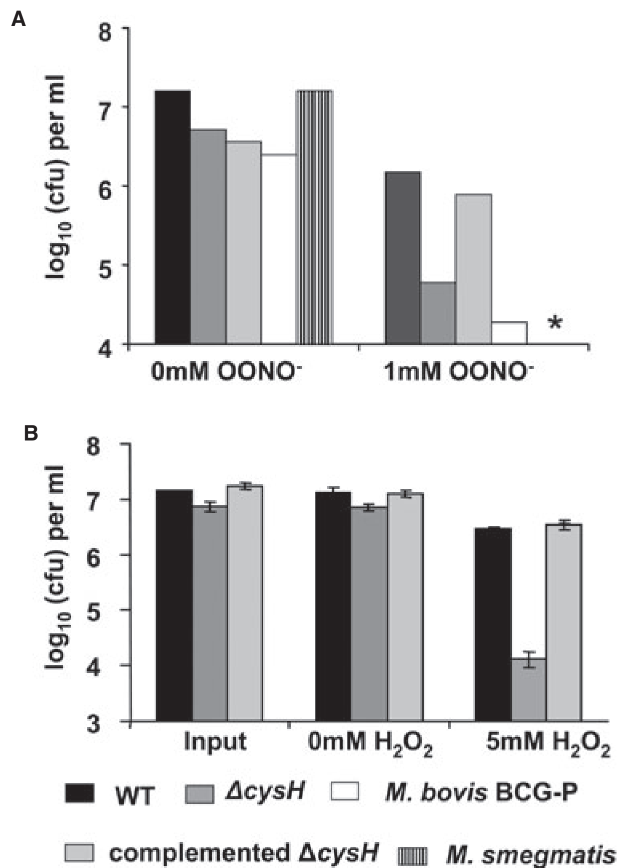

Fig. 6. CysH protects against RNI and ROI.
A. Survival of WT, Δ*cysH*, complemented Δ*cysH* (pMVGS:*cysH*) and BCG after exposure to 1 mM $ONOO^-$. (*) Only 10 cfu of *M. smegmatis* were detected after treatment with $ONOO^-$. This experiment was not done in triplicate; instead several different independent experiments were done (see Fig. S5). *M. smegmatis* and BCG were used as a control to measure the potency of $ONOO^-$.
B. Survival of WT, Δ*cysH* and complemented Δ*cysH* (pMVGS:*cysH*) after exposure to 5 mM H_2O_2 for 5 h. Error bars show mean ± SD of triplicate.

with WT) in NOS2$^{-/-}$ mice, we infected mice deficient in both NADPH oxidase and NOS2 (gp91phox$^{-/-}$ NOS2$^{-/-}$) activities. The gross lung pathology and lung tissue histology were almost identical among the two groups of mice, and the replication of the mutant strain was slightly faster than that of the WT (see Fig. S6). However, it was difficult to obtain large numbers of gp91phox$^{-/-}$ NOS2$^{-/-}$ mice to carry out a statistically viable survival study. A second complicating factor is that these mice must be maintained on two antibiotics to prevent spontaneous infections. Therefore, we used aminoguanidine (AG) to suppress the activity of NOS2 (Chan *et al.*, 1995) in gp91phox$^{-/-}$ mice, which are commercially available in large numbers, sufficient for statistically relevant studies to be undertaken. AG was given to gp91phox$^{-/-}$ (gp91phox$^{-/-}$AG) mice in drinking water *ad libitum*. Here, it was found that, extending the results seen for the gp91$^{phox-/-}$ NOS2$^{-/-}$ mice, lung tissue histology and the gross lung pathology were almost identical among the two groups of mice (Figs 2 and 5 see Table S1 and Fig. S4). The replications of WT and Δ*cysH* were almost identical in gp91phox$^{-/-}$AG (Fig. 7A). Also the survival of two groups of gp91phox$^{-/-}$AG mice infected with either Δ*cysH* or WT were very similar (Fig. 7B). These results further support the claim that CysH protects against both the effects of NADPH oxidase and NOS2, and may, in part, explain the relative attenuation of Δ*cysH* in NOS2$^{-/-}$ mice.

Discussion

This study provides evidence supporting the importance of the sulphur assimilation pathway in protection of Mtb

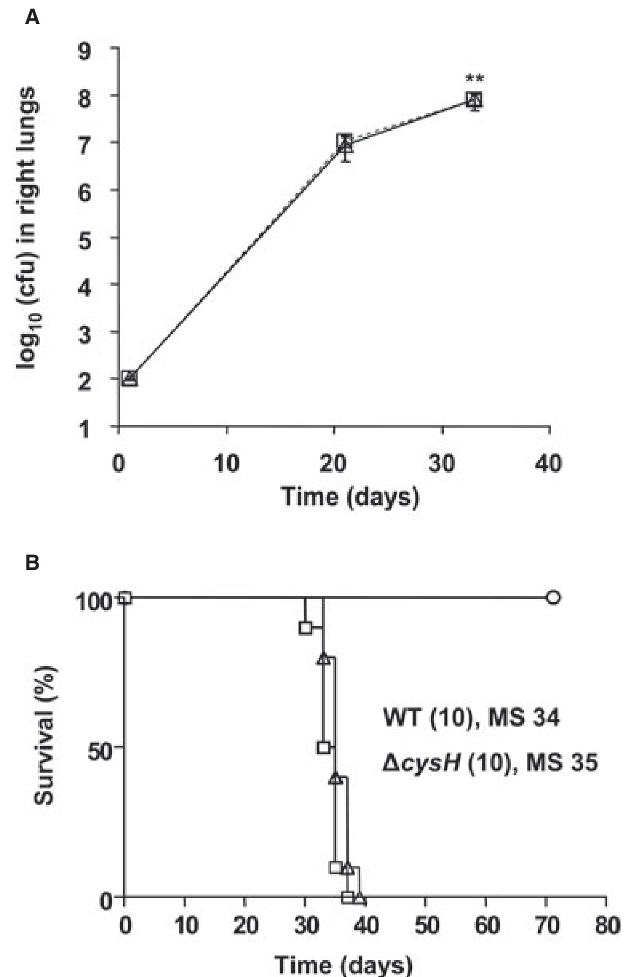

Fig. 7. Δ*cysH* is virulent in gp91phox$^{-/-}$AG mice.
A. Right lung cfu for Δ*cysH* (△) or WT (□ and dashed line) infected gp91phox$^{-/-}$AG mice after aerosol infection (approximately 100 bacilli per right lung) with WT (□), or Δ*cysH* (△), $n = 3$ mice per group at day 1 and $n = 4$ per group at other time points. Double asterisks indicate dying mice.
B. Survival of gp91phox$^{-/-}$AG mice (*n* in brackets and MS is median survival) infected as in (A). WT (□), Δ*cysH* (△), uninfected gp91phox$^{-/-}$AG mice control (○), $n = 7$.

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against the effects of RNI and ROI. The observed protection against $ONOO^-$ and H_2O_2 conferred by CysH is likely due to reductive scavenging by cysteine or downstream reduced-sulphur-containing metabolites. Cysteine is the source of sulphur for a variety of metabolites such as biotin, lipoic acid, molybdopterin, thiamine and mycothiol (Buchmeier *et al*., 2003; Loiseau *et al*., 2003). Lipoic acid is effective against oxidative stress *in vivo* and known to scavenge radical species like NO, superoxide and peroxides (Haramaki *et al*., 1997). Mycobacteria do not produce the low-molecular-mass thiol glutathione (which protects mammalian cells against oxidants). Instead, mycothiol appears to have functionally replaced glutathione in these organisms (Buchmeier *et al*., 2003) and mycothiol has been shown to protect mycobacteria against ROI (Rawat *et al*., 2002; Buchmeier *et al*., 2003). Also the mycothiol is the only major low-molecular-mass thiol in mycobacteria (Spies and Steenkamp, 1994).

We have previously shown that Δ*cysH* cannot utilize inorganic SO_4^{2-} to synthesize mycothiol (Mougous *et al*., 2002), and therefore must scavenge reduced sulphur for this purpose. Thus, the amounts of mycothiol that are present in Δ*cysH in vivo* could very well be restricted, and may limit protection against radicals and oxidants produced by the host immune system. In addition, it has been recently shown that sulphhydryl (thiol)-containing enzymes in Mtb can be nitrosylated by exposure to RNI and as a consequence their activity can be inhibited (Rhee *et al*., 2005). Therefore it is possible that Δ*cysH* is more susceptible to RNI than WT as Δ*cysH* (which lacks CysH) may possess fewer sulphhydryl-containing enzymes compared with WT. *In vitro* CysH is clearly important for primary amino acid metabolism, as Δ*cysH* is auxotrophic for cysteine and methionine. However, *in vivo*, CysH appears to be critical for bacterial survival only after the adaptive immune response is established in immunocompetent mice. Thus, the bacterium appears to have adapted a basic housekeeping function for defence against the immune system. Presumably, the downstream metabolic products of CysH protect the bacteria against the effects of NADPH oxidase and NOS2, similar to the protection previously ascribed to UvrB (Darwin and Nathan, 2005).

The prolonged survival of Δ*cysH*-infected $Rag1^{-/-}$ and $NOS2^{-/-}$ mice compared with the survival of the same groups of mice infected with the WT suggests that the mutant is partially attenuated in these immunodeficient mice. The observed susceptibility of Δ*cysH* to RNI and ROI demonstrates that the apparent attenuation of the mutant could be explained by its susceptibility to the residual effects of these products in these mice (ROI/RNI in $Rag1^{-/-}$ mice and ROI in $NOS2^{-/-}$ mice). Indeed, when both of these effects were eliminated by the use of $gp91phox^{-/-}NOS2^{-/-}$ mouse, or when the activity of NOS2 was suppressed by AG in $gp91phox^{-/-}$ mice, Δ*cysH* no longer showed attenuation compared with the WT. Thus, the requirement by Mtb of CysH during the adaptive immune phase may be attributed to its role in protection against RNI and ROI. The replication rates of the two strains were similar in immunocompetent mice during the first 19 days of infection. This suggests that the mutant is able to scavenge reduced-sulphur metabolites from the animal tissue, which enables it to defend against the early effects of RNI and ROI. However, as RNI and ROI levels progressively increase over the course of infection (Nathan *et al*., 1983; Ehrt *et al*., 2001; Shi *et al*., 2003) due to recruitment of pro-inflammatory cells, the ability to scavenge exogenous metabolites by the mutant may not be sufficient to overcome the effects of the higher concentrations of the effector molecules. Therefore, in the absence of CysH, Δ*cysH* may have to compete for the reduced sulphur that is available in the host tissues, and this competition may be responsible for the initial reduction of bacilli around 16–42 days pi. After the initial reduction, the remaining bacteria may enter an equilibrium population with the supply of reduced sulphur, so that their numbers stabilize over the time. The above hypothesis is further supported by our *in vitro* data that show that Δ*cysH* is more susceptible to H_2O_2 and $ONOO^-$ under reduced-sulphur starvation conditions. An alternative explanation could be that this initial reduction could simply be due to the down modulation of host immunity with time.

The ability of Mtb complex to scavenge reduced sulphur was confirmed by previous findings that two independent methionine auxotrophs of *M. bovis* BCG, lacking functional sulphate transport, were not compromised for survival in mice (McAdam *et al*., 1995; Wooff *et al*., 2002). Recently, Wheeler *et al*. (2005) have elucidated the biochemical pathway of transferring reduced sulphur from methionine to cysteine in Mtb and have estimated that Mtb can take up methionine five to six times faster than sulphate at physiological conditions. However, there are previous reports indicating the failure of several other Mtb amino acid auxotrophs to replicate in mouse tissues (McAdam *et al*., 1995; Hondalus *et al*., 2000; Pavelka *et al*., 2003). Therefore, an interesting question arises as to whether Mtb phagosome is selective in its permeability to amino acids or whether Mtb has access to reduced-sulphur-containing metabolite(s) other than to the amino acids, methionine and cysteine. The above question could be addressed by generating methionine and cysteine auxotrophic Mtb mutants by disrupting genes involved in the non-assimilatory pathway for methionine and cysteine biosynthesis.

In a previous study, a mutant in the sulphate transporter encoded by the gene *cysA* (first step in sulphur assimilation) could be rescued by supplementation with methionine but not with cysteine (Wooff *et al*., 2002). In contrast

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to this finding, we have shown that *cysH* mutants in both Mtb (this study: see Fig. S3) and *M. smegmatis* (Williams *et al*., 2002) can be rescued by either amino acid. It should be noted that a *cysA* mutant of *E. coli* can also be rescued by either amino acid (Wooff *et al*., 2002). Moreover, our findings are in accordance with the observation that methionine can be metabolically labelled in Mtb H37Rv with radiolabelled cysteine (Wheeler *et al*., 2005).

As RNI and ROI are two species that play a vital role in controlling other intracellular pathogens, it is possible that availability of reduced sulphur in bacteria plays a crucial role in intracellular pathogenesis. Therefore, further studies on the importance of acquisition of reduced sulphur by intracellular pathogens and the means of reduced-sulphur scavenging by Mtb from host tissues are warranted. Finally, this study suggests that therapeutics targeted at the acquisition of reduced sulphur by Mtb may prove effective at preventing persistence of this pathogen.

Experimental procedures

Mycobacteria and culture conditions

Wild-type Mtb H37Rv and its derivative strains, *M. bovis* BCG-P and *M. smegmatis*, were grown in Middlebrook 7H9 broth containing 10% ADC (Becton Dickinson and Company, Sparks, MD), 0.2% glycerol and 0.05% Tween 80 (7H9-ADCT) or on Middlebrook 7H10 agar containing OADC (Becton Dickinson and Company), 0.5% glycerol and antifungal agent cycloheximide (100 $\mu g\ ml^{-1}$) (Sigma-Aldrich). Antibiotics included hygromycin (50 $\mu g\ ml^{-1}$) and kanamycin (25 $\mu g\ ml^{-1}$). For the growth of Δ*cysH* all the above media were supplemented with 1–2 mM methionine. The presence or absence of the above amounts of methionine on 7H10 agar plates made no difference in recovery of WT cfu.

Single cell suspensions

Bacteria were passed through a 5 µm pore filter to prepare single cell suspensions prior to mouse infection, growth curves and *in vitro* assays described in this study.

Generation and the complementation of the Mtb H37Rv cysH *mutant*

The Mtb H37Rv Δ*cysH* was constructed by the method of Parish and Stoker (2000). Disrupted alleles were created by amplifying 2 kb regions upstream and downstream of the *cysH* gene. Following digestion with the appropriate restriction enzymes, these PCR products were subcloned into the p2NIL vector and subsequently interrupted by a hygromycin-resistance cassette (see Fig. S2). Mutant selection and additional vector information have been previously described (Parish and Stoker, 2000). Deletion of the gene was confirmed by the Southern blot analysis. Δ*cysH* was complemented by a copy of WT *cys*H in an extra chromosomal plasmid (pMGS) or in an integrative vector pMVGS. Construction of pMGS and pMVGS was described by Mougous *et al*. (2004). In brief, these two vectors contain the promoter region of the Mtb glutamine synthetase (Harth and Horwitz, 1997) cloned into the promoterless vectors pMS2.kan (Kaps *et al*., 2001) and pMV306.kan (Converse *et al*., 2003).

Mouse infections

Female C57BL/6, NOS2$^{-/-}$, Rag1$^{-/-}$ and gp91phox$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). gp91phox$^{-/-}$ NOS2$^{-/-}$ mice were a kind gift from Dr Carl F. Nathan. gp91phox$^{-/-}$ NOS2$^{-/-}$ mice were maintained on a medicated diet (Bio-serve, Frenchtown, NJ) containing itracoconazole and enrofloxacin, so that each mouse will receive an average of 1.2 and 0.12 mg of the drugs per day respectively. gp91phox$^{-/-}$ mice were given 2.5% (wt/vol) AG (Sigma) in sterile drinking water *ad libitum* as described by Chan *et al.* (1995) to inhibit NOS. Administration of AG in drinking water was initiated 24 h prior to infection. Seven- to 8-week-old mice were infected by either intravenous or aerosol route. Bacteria were aerosolized by Inhalation Exposure System (IES) (Glas-col, Terre Haute, IN) to deliver 100–200 (25 in one experiment) bacilli per right lung. For intravenous infections, mice were given 10^6 bacteria in 0.2 ml of phosphate-buffered saline containing 0.05% Tween 80 (PBST) into the lateral tail vein. In both methods of infection (with the exception of data presented in Fig. 1A), right lungs of three mice (per each infection per mouse strain) were harvested 24 h pi to determine the number of cfu per organ. Colony-forming units in each organ were determined by plating diluted (undiluted at 24 h after aerosol infection) organ homogenates on 7H10-OADC (with or without methionine) and counting colonies after 3–4 weeks.

Determination of mouse morbidity

Loss of weight accompanied by failure to groom, ruffled fur, and lethargy were used to determine the end-point of morbidity, in addition (in some instances) to the recommendation of the veterinary staff of North Animal Facility of UC Berkeley. Health of the mice was monitored daily by the above veterinary staff. Statistical analysis of survival curve was performed by Kaplan–Meier plot. Differences were considered significant at $P < 0.05$.

Histology

Mouse left lung fixed in 10% neutral (PBS) buffer formalin was embedded in paraffin, sectioned, and stained for histology with either haematoxylin and eosin (H&E) or the Ziehl-Neelsen technique. Sectioning and staining were done by Histology Consultation Services, Everson, Washington. For comparative purposes, sections were obtained from the same regions of all lungs; three sections were obtained from each lung. Sections obtained from the top and the bottom parts of the lung were H&E stained while the section obtained from the middle region was stained by the Ziehl–Neelsen technique.

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Pathology analysis

Pathology of each lung was assessed for two to four H&E stained sections and one to two sections stained by the Ziehl–Neelsen technique. Sections from three mice lungs were used per time point per Mtb strain for pathological analysis. Histopathological analysis was done by a veterinary pathologist from School of Veterinary Medicine UC Davis.

Susceptibility to peroxynitrite

Peroxynitrite assays were done as described by Yu *et al.* (1999). Bacteria were exposed to 1 mM $ONOO^-$ consecutively for seven to ten 3-min cycles with intermediate washing to remove $ONOO^-$. Surviving bacteria were enumerated by plating on 7H10 agar. For experiments involving depletion of the intracellular pool of reduced sulphur, Δ*cysH* was changed to 7H9-ADC without methionine, 24 h prior to the $ONOO^-$ susceptibility assays.

Susceptibility to H_2O_2

Before the start of the H_2O_2 assays, the medium was changed to the 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween-80, 0.5% bovine serum albumin, 0.2% dextrose and 0.085% sodium chloride (7H9-ADNacl). Bacteria were exposed to 5 mM H_2O_2 for 5 h, and then the surviving bacteria were enumerated by plating on 7H10 agar. For experiments involving depletion of the intracellular pool of reduced sulphur, Δ*cysH* was changed to 7H9-ADC without methionine, 24 h prior to the H_2O_2 susceptibility assays.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Sulphur assimilation pathway of Mtb.

Fig. S2. Southern blot analysis of Δ*cysH*.

Fig. S3. Δ*cysH* is auxotrophic for cysteine and methionine.

Fig. S4. Attenuation and virulence of Δ*cysH* by lung histology.

Fig. S5. Δ*cysH* is susceptible to $ONOO^-$.

Fig. S6. Cfu recovery and lung pathology of gp91phox$^{-/-}$ NOS2$^{-/-}$ mice.

Table S1. Comparison of the lesions and the bacteria in the lung tissues of different mouse strains by the grading system employed by the pathologist.

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