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The principal sigma factor *sigA* mediates enhanced growth of *Mycobacterium tuberculosis in vivo*

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Summary

The ability of *Mycobacterium tuberculosis* to grow in macrophages is central to its pathogenicity. We found previously that the widespread 210 strain of *M. tuberculosis* grew more rapidly than other strains in human macrophages. Because principal sigma factors influence virulence in some bacteria, we analysed mRNA expression of the principal sigma factor, *sigA*, in *M. tuberculosis* isolates during growth in human macrophages. Isolates of the 210 strain had higher *sigA* mRNA levels and higher intracellular growth rates, compared with other clinical strains and the laboratory strain H37Rv. *SigA* was also upregulated in the 210 isolate TB294 during growth in macrophages, compared with growth in broth. In contrast, H37Rv *sigA* mRNA levels did not change under these conditions. Overexpression of *sigA* enhanced growth of recombinant *M. tuberculosis* in macrophages and in lungs of mice after aerosol infection, whereas recombinant strains expressing antisense transcripts to *sigA* showed decreased growth in both models. In the presence of superoxide, sense *sigA* transformants showed greater resistance than vector controls, and the antisense *sigA* transformant did not grow. We conclude that *M. tuberculosis sigA* modulates the expression of genes that contribute to virulence, enhancing growth in human macrophages and during the early phases of pulmonary infection *in vivo*. This effect may be mediated in part by increased resistance to reactive oxygen intermediates.

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Introduction

Recent evidence points to significant variation in the pathogenic potential of individual *Mycobacterium tuberculosis* strains. Manca *et al.* (2001) reported that two *M. tuberculosis* isolates differed markedly in their capacity to cause rapidly fatal disease and to elicit a type 1 cytokine response in mice. Molecular epidemiological studies have also shown that a small percentage of *M. tuberculosis* strains cause a large proportion of cases (Small *et al.*, 1994; Barnes *et al.*, 1997; Bishai *et al.*, 1998; Van Soolingen *et al.*, 1999), implying that some strains spread more effectively than others. Some of the most widespread *M. tuberculosis* strains are those of the Beijing/W family (Van Soolingen *et al.*, 1995; Bifani *et al.*, 1999; 2002). The *M. tuberculosis* 210 strain is a member of the Beijing family that contains 21 copies of the insertion element IS*6110* and is widely distributed in the south-west and south-central United States (Barnes *et al.*, 1997; Yang *et al.*, 1998; Weis *et al.*, 2002). Isolates of the 210 strain that infected different patients grew more rapidly than other strains in human macrophages, suggesting an enhanced ability to avoid host defences (Zhang *et al.*, 1999).

The capacity of *M. tuberculosis* to grow in human mononuclear phagocytes is a central feature of its pathogenic potential, and identification of mycobacterial genes that contribute to intracellular mycobacterial growth is an area of intensive investigation. Deletion of a variety of *M. tuberculosis* genes reduces intracellular growth rates (Berthet *et al.*, 1998; De Voss *et al.*, 1998; Perez *et al.*, 1998; Buchmeier *et al.*, 2000), and expression of other genes is increased when *M. tuberculosis* grows intracellularly, rather than in broth (Yuan *et al.*, 1998; Graham and Clark-Curtiss, 1999; Dubnau *et al.*, 2002). However, the mycobacterial genes that mediate the enhanced intracellular growth rates of some *M. tuberculosis* strains remain unidentified.

Principal sigma factors primarily regulate housekeeping genes in bacteria but, in some species, they also regulate the expression of specific virulence genes (Puente *et al.*, 1996; Steffen *et al.*, 1997; Lohrke *et al.*, 1999; Fraser *et al.*, 2002) and may be induced in response to stress

(Allen *et al*., 1998; Aramaki and Fujita, 1999; Liao *et al*., 1999). Evidence from another member of the *M. tuberculosis* complex suggested that the *M. tuberculosis* sigma factor, *sigA*, also known as *rpoV* (Collins *et al*., 1995) and Rv2703 (Cole *et al*., 1998), also played a role in virulence. In *Mycobacterium bovis*, a point mutation in *sigA* resulted in markedly attenuated virulence for guinea pigs (Collins *et al*., 1995). Therefore, we wished to determine whether the principal sigma factor, *sigA*, contributes to the capacity of the 210 strain to grow more rapidly than other *M. tuberculosis* strains in human mononuclear phagocytes. To address this issue, we compared growth rates of strain 210 isolates and other *M. tuberculosis* isolates in human mononuclear phagocytes with *sigA* expression levels. We also studied the capacity of *M. tuberculosis* transformants expressing different levels of *sigA* mRNA to grow in human mononuclear phagocytes and in mice.

Results

Mycobacterial growth and gene expression of the 210 strain and H37Rv in mononuclear phagocytes

We demonstrated previously that isolates of the 210 strain grow more rapidly than other clinical *M. tuberculosis* strains in monocyte-derived macrophages (Zhang *et al*., 1999). In six experiments, a 210 strain isolate TB294 consistently grew more rapidly in monocyte-derived macrophages than H37Rv (mean growth index 208 ± 42 versus 24.5 ± 3, $P < 0.001$; Fig. 1A). Both isolates grew at the same rates in 7H9 broth (data not shown).

To determine whether the higher growth rate of TB294 was associated with enhanced expression of *sigA*, we cultured TB294 and H37Rv in monocyte-derived macrophages for 6 days, and used real-time polymerase chain reaction (PCR) to measure *sigA* mRNA expression, normalized for 16S rRNA content. mRNA levels for *sigA* were 10-fold higher in intracellular TB294 than in intracellular H37Rv ($P = 0.02$, Fig. 1B). To determine whether this increase reflected a generalized upregulation of gene expression in TB294, we measured mRNA expression of another sigma factor, *sigB*, and the gene *mce1*, which is thought to regulate mycobacterial entry into human cells (Arruda *et al*., 1993). We expected that mRNA expression of *mce1* would not be increased in TB294 because its capacity to enter mononuclear phagocytes is not enhanced, compared with other *M. tuberculosis* strains (Zhang *et al*., 1999). Expression of mRNA for *sigB* and *mce1* was similar in H37Rv and TB294 (Fig. 1B). As an additional control, we measured the expression of 23S rRNA and found similar levels of expression in the two strains. These findings demonstrate that *sigA* mRNA expression is greater in TB294 than in H37Rv when they are cultured in macrophages.

We next wanted to determine whether the higher levels of *sigA* mRNA in TB294 in macrophages were specific for intracellular growth. Therefore, we compared *sigA* mRNA levels, normalized for 16S rRNA content, in TB294 and H37Rv, cultured in either 7H9 medium until mid-log phase or the human monocytic cell line MonoMac6. Results were expressed as the ratio of *sigA* mRNA in MonoMac6 cells compared with that in 7H9 medium. *SigA* expression of H37Rv was similar in 7H9 medium and during intracellular growth (Fig. 1C), confirming previous reports (Dubnau *et al*., 2002). In contrast, *sigA* mRNA levels of TB294 were increased 3.5-fold after 1 day of growth in MonoMac6 cells, and this increased to 7.4-fold after 6 days. Therefore, compared with organisms cultured in broth, *sigA* mRNA expression is upregulated in intracellular TB294 but not in intracellular H37Rv.

sigA *expression in a panel of* M. tuberculosis *strains growing in mononuclear phagocytes*

We evaluated four isolates that were members of the 210 strain. Each isolate was obtained from a different patient, none of whom was epidemiologically linked by disease transmission. In addition, we studied four isolates from patients in central Los Angeles with extensive pulmonary tuberculosis who spent long periods at homeless shelters while they were infectious. Despite ample opportunity for disease transmission, these isolates had restriction fragment length polymorphism (RFLP) patterns that were not shared by isolates from any other tuberculosis patient in central Los Angeles, suggesting that these strains were limited in their potential for transmission (Barnes *et al*., 1997). To determine whether the growth rates of these *M. tuberculosis* strains correlated with expression of *sigA* mRNA, we infected MonoMac6 cells with each of these eight clinical isolates or with H37Rv. MonoMac6 cells were used instead of monocyte-derived macrophages, because it was not feasible to obtain sufficient numbers of monocyte-derived macrophages from a single donor to permit infection with nine different strains and isolation of adequate mycobacterial mRNA for analysis. Mycobacterial growth was measured after 10 days, and mRNA expression of *sigA* in each strain was determined by real-time PCR, expressed as a ratio of *sigA* gene expression for the clinical strain compared with H37Rv. We confirmed our previous findings that 210 strain isolates grew faster than other clinical isolates (mean growth index 85 ± 6 versus 42 ± 6, $P = 0.002$; Fig. 2). All clinical strains showed enhanced expression of *sigA* compared with H37Rv, and expression of *sigA* mRNA was greater in the 210 strains than in the other clinical strains (8.0 ± 2.1 versus 1.9 ± 0.3, $P < 0.05$). *sigA* mRNA expression correlated strongly with the growth index (correlation coefficient = 0.84).

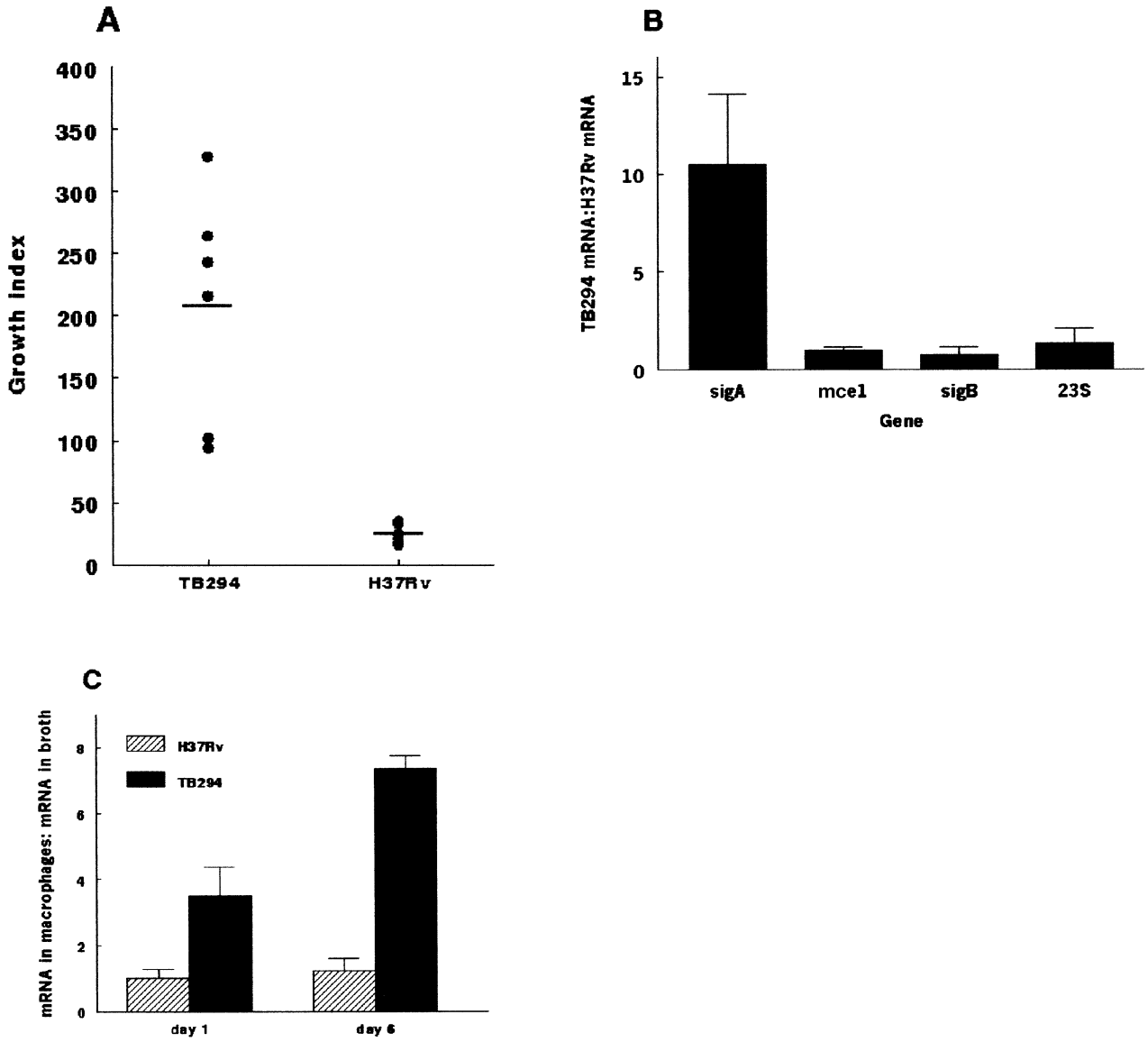

Fig. 1. Growth and mycobacterial gene expression of TB294 and H37Rv in mononuclear phagocytes.
A. Monocyte-derived macrophages from six normal donors were each infected with TB294 or H37Rv at an MOI of 1:50. After 10 days, macrophage lysates were diluted and plated, and cfu were measured. The growth index was calculated as cfu at day 10 divided by cfu at day 1. Horizontal lines show the mean values.
B. Monocyte-derived macrophages from four normal donors were each infected with TB294 or H37Rv at an MOI of 1:4. After 6 days, mRNA expression of each gene was quantified by real-time PCR, normalized for 16S rRNA content. mRNA expression for each H37Rv gene was arbitrarily given a value of 1, and the values show the ratio of TB294 mRNA expression compared with H37Rv mRNA expression for each gene. Mean values and standard errors are shown.
C. TB294 and H37Rv were cultured in 7H9 medium until mid-log phase (OD_{600} of 0.4–0.7) or in the human monocytic cell line MonoMac6 for 1 or 6 days. *SigA* mRNA expression was quantified by real-time PCR, after normalization for 16S rRNA content. Results are expressed as the ratio of *sigA* mRNA levels in MonoMac6 cells to *sigA* mRNA levels in 7H9 medium. Mean values and standard errors for three or four experiments are shown.

Intracellular growth of TB294 and H37Rv carrying sense or antisense sigA *plasmids*

The above experiments suggest that increased *sigA* expression is associated with enhanced intracellular growth of *M. tuberculosis*. To determine definitively whether higher *sigA* mRNA levels can increase intracellular growth, we used *sigA* sense and antisense plasmids to alter gene expression in H37Rv and TB294. We first amplified the full-length 1584 bp *sigA* gene from genomic DNA of TB294 by PCR. The DNA sequence was identical to that for *sigA* of H37Rv (Cole *et al.*, 1998; http://genolist.pasteur.fr/TubercuList). Transformants of TB294 and H37Rv bearing sense and antisense *sigA* plasmids or

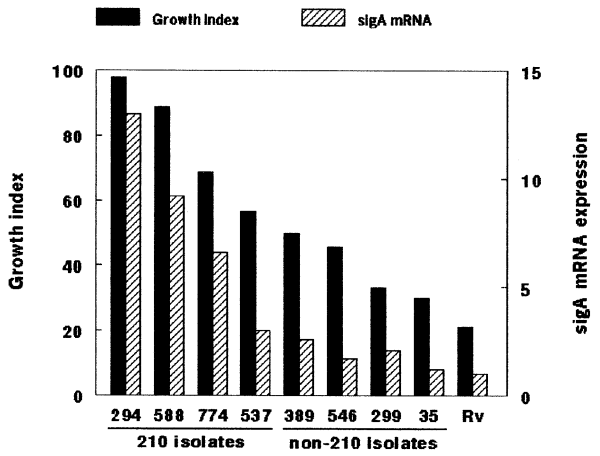

Fig. 2. Growth and *sigA* mRNA expression of nine *M. tuberculosis* strains in MonoMac6 cells. MonoMac6 cells were infected with four isolates of the 210 strain, four other clinical isolates and H37Rv at an MOI of 1:4, and cfu were measured after 10 days. The growth index was calculated as cfu at day 10 divided by cfu at day 1. The mean values of two experiments for each strain are shown. After 6 days of culture, *sigA* mRNA expression was quantified by real-time PCR, normalized for 16S rRNA content. *sigA* mRNA expression for H37Rv was arbitrarily given a value of 1, and the values for other isolates are shown as a ratio compared with H37Rv.

vector control plasmids were constructed, as outlined in *Experimental procedures*.

The TB294 and H37Rv transformants grew at similar rates in broth (data not shown). However, the sense *sigA* TB294 and H37Rv transformants grew 7–15 times more rapidly than the corresponding vector control strains ($P = 0.05$ and 0.03 respectively; Fig. 3). In contrast, the growth indices of the antisense *sigA* transformants were only 1/16th to one-fifth of those of the vector control strains ($P = 0.002$ and 0.01; Fig. 3). Insertion of the sense *sigA* construct into H37Rv resulted in intracellular growth rates comparable to those of the TB294 vector control, whereas insertion of the antisense *sigA* construct into TB294 reduced the intracellular growth rate to that of the H37Rv vector control. These results demonstrate that upregulation of *sigA* mRNA enhances intracellular mycobacterial growth.

Protein levels of sigA *in sense and antisense* sigA *transformants growing in human mononuclear phagocytes*

To verify that sense and antisense transcripts were influencing *sigA* protein levels, we cultured the TB294 and H37Rv *sigA* transformants in MonoMac6 cells. After 6 days, cells were lysed, proteins were extracted, and Western blotting was performed with 2G10, a monoclonal antibody (mAb) to the σ^{70} subunit of *Escherichia coli* RNA polymerase, which recognizes *sigA* of *Mycobacterium smegmatis* (Strickland *et al.*, 1988; Predich *et al.*, 1995).

The *M. tuberculosis sigA* has a predicted molecular mass of 58 kDa, but runs as a doublet of 85 and 87 kDa on the 10% polyacrylamide gel (Fig. 4A). The σ^{70} subunit of *E. coli* and *sigA* of *M. smegmatis* have predicted molecular weights of 69 and 52 kDa respectively. They also run at higher apparent molecular weights of 93 and 79 kDa

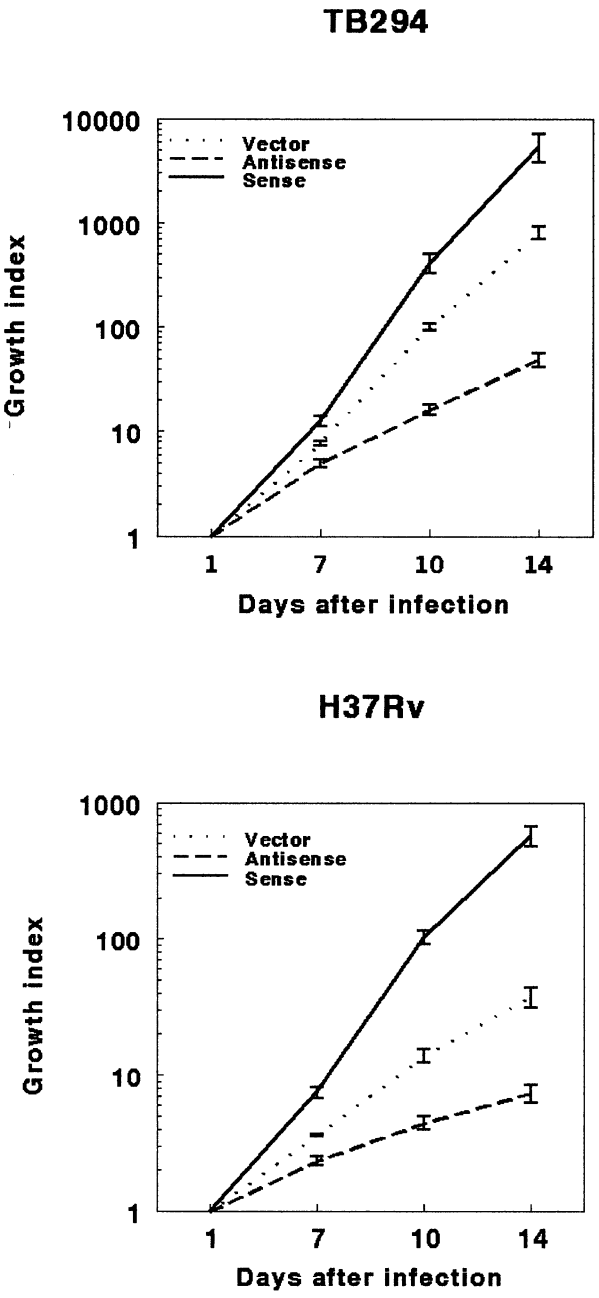

Fig. 3. Intracellular growth of sense and antisense *sigA* transformants of TB294 and H37Rv. MonoMac6 cells were infected with transformants bearing sense *sigA*, antisense *sigA* or vector control plasmids at an MOI of 1:50, and cfu were measured at 7, 10 and 14 days. Results are expressed as a growth index, calculated by dividing cfu at the time point shown by cfu at day 1. The means and standard errors for three experiments are shown.

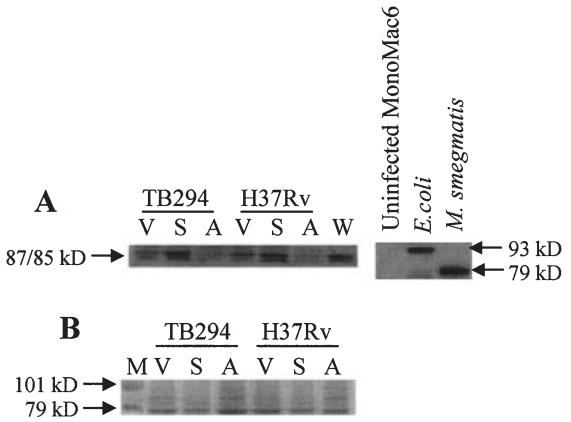

Fig. 4. SigA protein levels of TB294 and H37Rv transformants during growth in MonoMac6 cells.
A. MonoMac6 cells were infected with TB294 or H37Rv bearing sense *sigA* (S), antisense *sigA* (A) or vector control (V) plasmids at an MOI of 1:1. After 6 days, cells were lysed, and proteins were extracted. Protein extracts were also prepared from uninfected MonoMac6 cells, wild-type TB294 (W) and *M. smegmatis* cultured in 7H9 and *E. coli* cultured in Luria–Bertani medium. Protein extracts were run on a 10% reducing SDS polyacrylamide gel. The amount of extract in each lane was 50 μg for transformants cultured intracellularly, 25 μg for TB294 cultured *in vitro* and 20 μg for *M. smegmatis*, *E. coli* and uninfected MonoMac6 cells. Western blotting was performed with 2G10, a mAb to the σ^{70} subunit of *E. coli* RNA polymerase.
B. Protein extracts (50 μg per lane) obtained from MonoMac6 cells infected with each of the six transformants in (A) were run on a 10% reducing SDS polyacrylamide gel and stained with Coomassie blue. M = molecular weight markers.

respectively (Fig. 4A). Similar changes in mobility have been reported for mycobacterial and other sigma factors, perhaps because of their acidic nature or because they assume a more extended conformation on the gel (Strickland *et al.*, 1988; Predich *et al.*, 1995).

The *sigA* protein levels in the sense TB294 and H37Rv transformants were higher than those in the corresponding vector controls, whereas *sigA* levels were reduced in the antisense transformants (Fig. 4A). Protein loading in each lane was similar, as shown by staining with Coomassie blue (Fig. 4B). This indicates that the alterations in *sigA* mRNA expression induced by the sense and antisense transformants are reflected by changes in *sigA* protein levels.

Effect of superoxide and acid stress on growth of H37Rv, TB294 and TB294 transformants

During growth in macrophages, mycobacteria may encounter stress as a result of release of superoxide radicals or acidification of the phagosomal vacuole. To determine whether upregulation of *sigA* contributed to resistance to these stresses, we cultured H37Rv and TB294 in the presence of different concentrations of menadione, which generates superoxide anions (Garbe *et al.*, 1999). In the presence of 25 mM menadione, TB294 and H37Rv grew at similar rates. However, at concentrations ranging from 50 to 100 mM, TB294 grew more rapidly than H37Rv (Fig. 5, top; data not shown). In the presence of 70 mM menadione, TB294 grew slowly over 7 days, whereas H37Rv showed no growth. When the TB294 transformants were cultured with 70 mM menadione, the sense *sigA* transformant grew more rapidly than the vector control, and the antisense *sigA* transformant showed no growth (Fig. 5, bottom). In the absence of menadione, the transformants had comparable growth rates. Measurements of colony-forming units (cfu) closely paralleled those of optical density (data not shown).

The sense *sigA*, antisense *sigA* and vector control transformants showed no differences in growth rates at a pH of 4.2 or 5.5 over 4 days (data not shown).

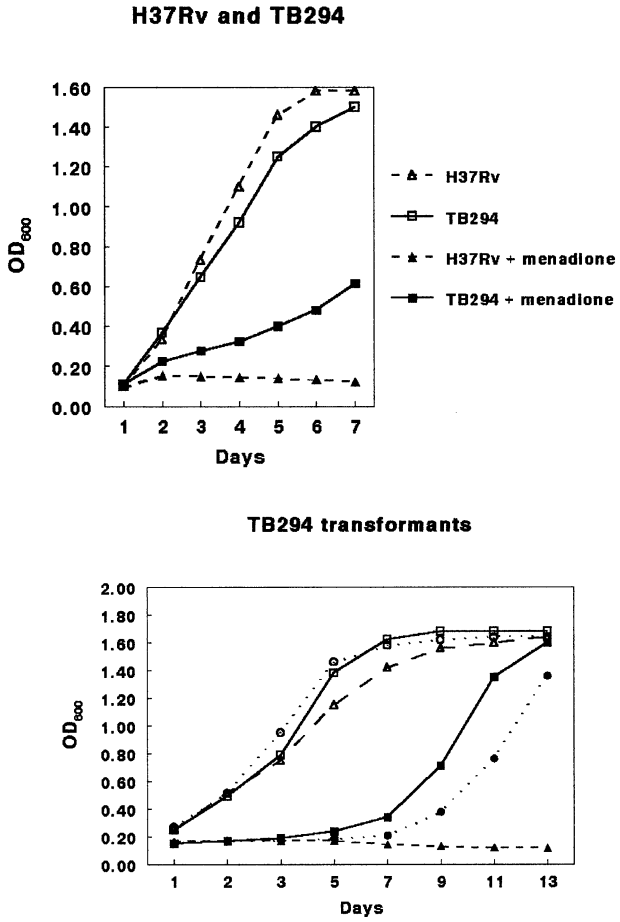

Fig. 5. Effect of superoxide stress on growth of H37Rv, TB294 and TB294 transformants. Top, growth of H37Rv and TB294 in the presence or absence of 70 μM menadione. Bottom, growth of TB294 transformants in the presence (closed symbols) or absence (open symbols) of 70 μM menadione. Squares with solid lines show the sense *sigA* transformant, circles with dotted lines show the vector control, and triangles with dashed lines show the antisense *sigA* transformant. Two experiments yielded similar results, and one is shown.

Growth of sense and antisense sigA *transformants of TB294 and H37Rv during initial pulmonary infection in mice*

Because the capacity of *M. tuberculosis* to grow in mononuclear phagocytes may not reflect its ability to survive in the host *in vivo*, we infected C57Bl/6 mice by aerosol with low doses of wild-type TB294, wild-type H37Rv and their transformants, and measured cfu during the first 20 days, before infection is controlled by the immune response. In preliminary experiments with inocula of 100–200 organisms, we found that, 20 days after infection, animals infected with TB294 had $9.1 \pm 0.3 \times 10^6$ cfu per lung, compared with $7.2 \pm 0.2 \times 10^5$ cfu per lung for animals infected with H37Rv ($P = 0.001$). We next infected animals with transformants of TB294. In two experiments, the sense *sigA* and antisense *sigA* transformants grew more rapidly and more slowly, respectively, compared with the vector control (Fig. 6). In experiment 1, cfu of the sense *sigA* transformant at day 20 was half a log greater than that of the vector control ($P < 0.001$, Fig. 6A), and cfu of the antisense *sigA* transformant was half a log less than that of the vector control ($P < 0.001$). In experiment 2, cfu of the sense *sigA* transformant at day 20 was 0.3 logs higher than that of the vector control ($P = 0.06$, Fig. 6B), and cfu of the antisense *sigA* transformant was 2.1 logs less than that of the vector control ($P < 0.001$). Compared with the vector control, the sense *sigA* transformant did not show markedly enhanced growth, perhaps because TB294 already produces relatively high levels of *sigA*. We therefore repeated these experiments by infecting mice with the H37Rv vector control and sense *sigA* transformant. cfu for the sense *sigA* transformant was 2.2 logs higher than that of the vector control ($P < 0.001$, Fig. 6C). These findings indicate that enhanced expression of *sigA* increases the capacity of *M. tuberculosis* to grow in mice during the initial phase of pulmonary infection.

Pulmonary histopathology during infection by sense and antisense sigA *transformants of TB294*

To evaluate the local host response to infection with the sense and antisense *sigA* transformants, lung sections from infected mice were obtained. In animals infected with the sense *sigA* and the vector control, there were coalescent inflammatory foci composed of macrophages, lym-

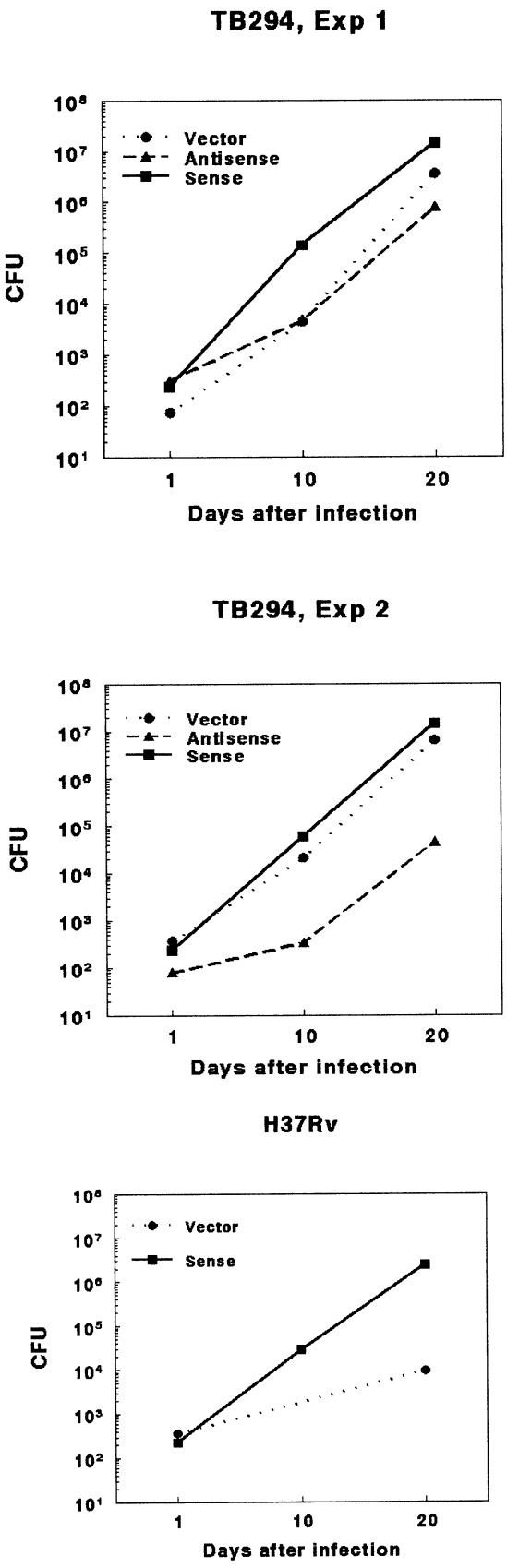

Fig. 6. Growth of sense and antisense *sigA* transformants in the lungs of mice. C57Bl/6 mice were infected by aerosol with ≈100–400 bacilli of the TB294 vector control, sense or antisense *sigA* transformants (top two graphs) or the H37Rv control or sense *sigA* transformant (bottom graph). cfu in lung homogenates were determined at days 1, 10 and 20. Each point represents the mean cfu from four to seven mice. Standard errors were less than 5% and are too small to be visible in the figure.

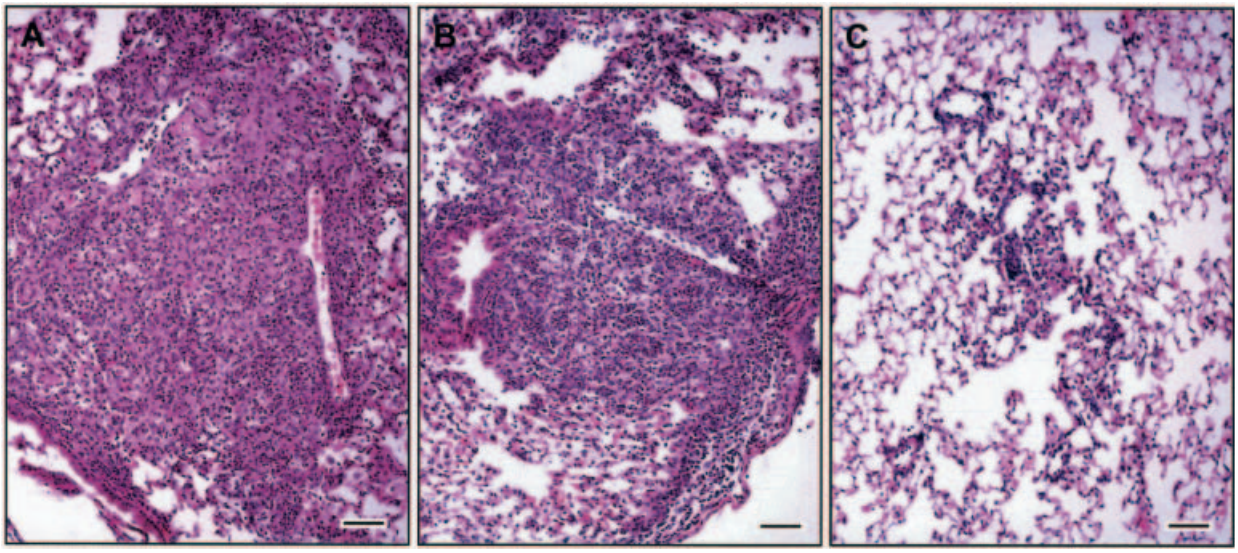

Fig. 7. Histological findings in lungs from mice challenged by aerosol with the sense *sigA* (A), vector control (B) and antisense *sigA* (C) transformants of *M. tuberculosis* TB294. Paraffin-embedded, formalin-fixed sections of mouse lung were obtained 20 days after infection and stained with haematoxylin and eosin. The scale bar = 100 μm.

phocytes, plasma cells and neutrophils, effacing the pulmonary parenchyma surrounding the bronchioles and vasculature (Fig. 7A and B). In contrast, minimal perivascular and peribronchiolar infiltrates of lymphocytes and plasma cells were scattered randomly throughout the parenchyma in animals infected with the antisense *sigA* transformant (Fig. 7C). This demonstrates that the inflammatory response to the antisense *sigA* transformant was significantly reduced, consistent with reduced bacillary growth.

Discussion

We found that the principal sigma factor influences a central pathogenic property of *M. tuberculosis*, that is, the ability to multiply in host cells and in the lungs. *M. tuberculosis* transformants bearing sense or antisense *sigA* plasmids or vector control plasmids had similar growth rates *in vitro*. However, transformants that overexpressed *sigA* grew more rapidly in mononuclear phagocytes and in C57Bl/6 mice compared with control strains. Conversely, transformants expressing antisense *sigA* grew more slowly in host cells and in mice, and elicited a minimal local inflammatory response. In the presence of superoxide, sense *sigA* transformants showed greater resistance than vector controls, and antisense *sigA* transformants did not grow. The clinical isolate TB294, but not H37Rv, upregulated *sigA* expression during intracellular infection. In addition, there was a strong correlation between *sigA* mRNA levels and intracellular growth rates of clinical *M. tuberculosis* strains. These findings suggest that *sigA* controls the expression of genes that are critical for *M. tuberculosis* to grow intracellularly and in the lungs during the initial phase of infection *in vivo*, and that this is mediated in part by enhanced resistance to reactive oxygen intermediates.

Bacterial gene expression is controlled primarily at the level of gene transcription, and the specificity of transcript initiation is regulated by sigma factors, which interact with the core enzyme of RNA polymerase, permitting recognition and binding to specific promoters. *SigA* is considered to be the principal mycobacterial sigma factor because it is highly homologous to other principal sigma factors of the σ^{70} family and is an essential gene (Gomez *et al*., 1998). Like other primary sigma factors, *sigA* is believed to control the transcription of many housekeeping genes. In contrast, alternative sigma factors are thought to control genes required to adapt to specific conditions, such as heat or oxidative stress, as they are often induced in response to these conditions. For example, *M. tuberculosis sigE* and *sigH* are induced by heat stress (Manganelli *et al*., 1999, 2001; 2002) and during infection of mononuclear phagocytes (Graham and Clark-Curtiss, 1999). *M. tuberculosis sigE* and *sigH* deletion mutants are less tolerant to heat (Manganelli *et al*., 2001; 2002; Raman *et al*., 2001) and have a reduced capacity to grow in macrophages and to cause mortality in mice respectively (Manganelli *et al*., 2001; Kaushal *et al*., 2002), suggesting that these sigma factors induce the expression of genes that are important for interactions with the host. Comparison of gene expression between the mutant and parent strains suggests that the effects of *sigE* and *sigH* are mediated in part through the induction of other sigma factors, as well

as through direct control of potential virulence factors (Manganelli *et al*., 2001; 2002)

sigA mRNA levels were increased in intracellular TB294, indicating that, in some clinical strains, *sigA* expression can be altered by external stimuli. Stress conditions can alter levels of the principal sigma factor in other bacteria. For example, transcription of the principal sigma factors of *Pseudomonas aeruginosa* and *Bacillus subtilis* is increased during heat shock through recognition of the promoter by a stress-responsive alternative sigma factor (Allen *et al*., 1998; Aramaki *et al*., 1999; Liao *et al*., 1999). Upstream of the transcriptional start site for *M. tuberculosis sigA* (Gomez *et al*., 1998), we found the sequences 'gggct' and 'agtta', which closely resemble the –35 and –10 consensus sequences recognized by *sigE* (Manganelli *et al*., 2001), suggesting that this and perhaps other alternative sigma factors can regulate *sigA* expression. We speculate that one or more stress-responsive sigma factors may increase the transcription of *sigA* after entry into macrophages.

We believe that the increased *sigA* mRNA levels in 210 strain isolates during intracellular growth result from enhanced transcription of *sigA*. Similar findings could result from increased stability of *sigA* transcripts, but this is less likely as transcript stabilization is most marked in stationary phase (Albertson and Nystrom, 1994; Gomez *et al*., 1998), whereas *M. tuberculosis* divides actively in mononuclear phagocytes.

Based on our results, we hypothesize that *sigA* modulates not only essential genes, but also directly or indirectly regulates the expression of genes that facilitate intracellular survival and intrapulmonary growth of *M. tuberculosis*. This hypothesis is supported by several findings. First, transformants that overexpressed *sigA* had enhanced resistance to superoxide stress, suggesting that *sigA* controls oxidative stress response genes such as superoxide dismutase and *ahpC*. These results are similar to those reported for *M. tuberculosis sigE* and *sigH* deletion mutants, which showed increased susceptibility to oxidative stress (Manganelli *et al*., 2001; 2002; Raman *et al*., 2001). Secondly, a point mutation in *sigA* of *M. bovis* did not affect its ability to grow *in vitro* but markedly attenuated its virulence in guinea pigs (Collins *et al*., 1995), probably because of reduced expression of virulence genes that are activated by interactions between the putative transcription factor *whiB3* and *sigA* (Steyn *et al*., 2002). Thirdly, a variety of virulence genes in Gram-negative bacteria are controlled by principal sigma factors (Puente *et al*., 1996; Steffen *et al*., 1997; Lohrke *et al*., 1999; Fraser *et al*., 2002), sometimes in concert with additional regulatory factors. For example, binding of a bacterial signal transduction protein to promoters of virulence genes of *Bordetella pertussis* permits recognition of these same promoters by the σ^{70} RNA polymerase.

We found that *sigA* mRNA levels in H37Rv were similar in macrophages and in broth, confirming previous reports that *sigA* expression in this strain does not change during exposure to most stresses *in vitro* (Hu and Coates, 1999; Manganelli *et al*., 1999) or during intracellular growth (Dubnau *et al*., 2002). It is intriguing to speculate that the capacity to enhance *sigA* expression may have been lost during decades of laboratory propagation of H37Rv. Although there was no change in *sigA* mRNA in wild-type H37Rv, the increased intracellular growth rate of the H37Rv sense *sigA* transformant demonstrated that the downstream genes controlled by *sigA* that facilitate intracellular growth are fully active in H37Rv.

Recent evidence indicates that clinical isolates of *M. tuberculosis* exhibit considerably greater genetic variation than was previously believed (Fleishmann *et al*., 2002), but the impact of these differences on the transmission potential of *M. tuberculosis* strains is unknown. Molecular epidemiological studies have demonstrated that a small percentage of *M. tuberculosis* strains cause a large proportion of cases (Small *et al*., 1994; Barnes *et al*., 1997; Bishai *et al*., 1998; Yang *et al*., 1998; Van Soolingen *et al*., 1999), suggesting that individual strains differ in their capacity to spread in the population. However, few characteristics of individual strains have been identified that may contribute to these differences. The widespread C strain in New York City was more resistant to reactive nitrogen intermediates than other *M. tuberculosis* strains, perhaps favouring its survival in macrophages (Friedman *et al*., 1997). Two clinical *M. tuberculosis* strains also differed markedly in their virulence for mice and in their capacity to elicit a Th1 cytokine response (Manca *et al*., 2001). Our findings suggest that altered *sigA* mRNA expression represents another mechanism by which *M. tuberculosis* strains differ in their pathogenic potential.

The 210 strain is a member of the Beijing family of strains, which has caused outbreaks in many parts of the world (Yang *et al*., 1998; Bifani *et al*., 1999; Caminero *et al*., 2001) and is the dominant family of *M. tuberculosis* strains in Asia and North America (Van Soolingen *et al*., 1995; Bifani *et al*., 1999; 2002). The Beijing family strains may be widely distributed because they were introduced into multiple locations before other strains and have had more time to spread in these communities. However, one recent study showed that, after the introduction of a single Beijing isolate to an island, it spread rapidly and accounted for 27% of the tuberculosis cases on the island after 3 years (Caminero *et al*., 2001). This suggests that some Beijing isolates may spread more readily in human populations. Based on our current findings, we speculate that upregulation of *sigA* in the 210 strain enhances its growth in macrophages and in the lungs during the initial phase of infection, facilitating its capacity to spread in human populations.

Experimental procedures

Mycobacterium tuberculosis *strains and culture conditions*

We used isolates from eight tuberculosis patients evaluated in a molecular epidemiological study in Los Angeles (Barnes *et al*., 1997). Strains were examined by RFLP analysis, based on the distribution of the insertion sequence IS*6110*. TB294, TB537, TB588 and TB774 were members of the 210 strain and were isolated from patients that were not epidemiologically related (Barnes *et al*., 1997; Yang *et al*., 1998). TB299, TB546, TB389 and TB35 were obtained from patients who were infected with organisms that were not members of the 210 strain, as determined by RFLP analysis. H37Rv was obtained from the Sanger Center. Mycobacteria were grown without shaking at 37°C in 5% CO_2 in Middlebrook 7H9 medium (Difco) containing 10% oleic acid–albumin–dextrose–catalase (OADC; Difco) and either 0.2% glycerol or 0.05% Tween 80. Transformant strains were cultured with 20 mg ml^{-1} kanamycin sulphate.

Infection of human mononuclear phagocytes

Peripheral blood mononuclear cells were isolated from healthy tuberculin-negative donors by differential centrifugation on Ficoll-Paque PLUS (Amersham Biosciences), and 5×10^6 cells per well were plated in 24-well plates in RPMI-1640 containing 10% heat-inactivated human serum. After 2 h, non-adherent cells were removed, and adherent cells were cultured in 1 ml of Macrophage serum-free medium (Gibco) for 3–5 days to mature into macrophages before infection with *M. tuberculosis*. At this point, each well contained $\approx 5 \times 10^5$ cells, with >95% viability, based on Trypan blue staining. The human monocytic cell line MonoMac6 (Ziegler-Heitbrock *et al*., 1987) was cultured in RPMI containing 10% heat-inactivated fetal bovine serum, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 9 mg ml^{-1} bovine insulin.

In all experiments, monocyte-derived macrophages and MonoMac6 cells were infected with single-cell suspensions of *M. tuberculosis* in RPMI containing 10% heat-inactivated human serum at multiplicities of infection (MOIs) ranging from 1:50 to 1:1, as described previously (Zhang *et al*., 1998). At different times after infection, the supernatant was aspirated, macrophages were lysed with distilled water for 10 min, then incubated with 0.09% SDS for 10 min, and 20% bovine serum albumin (BSA) was added. Bacterial suspensions in cell lysates and supernatants were ultrasonically dispersed, serially diluted and plated in triplicate at 37°C on 7H10 agar (Difco) supplemented with 10% OADC and 0.5% glycerol. After 3 weeks, cfu were counted. cfu in supernatants were always <10% of cfu in the corresponding lysates, and the latter were recorded as a measure of intracellular mycobacteria.

Growth of mycobacteria under superoxide and acid stress

Mycobacterium tuberculosis was grown in 7H9 medium at 37°C to an OD_{600} of 0.1–0.2. Cultures were then aliquoted, and menadione (2-methyl-1,4-naphthoquinone; Sigma) was added to triplicate aliquots at concentrations ranging from 25 to 100 mM. In other experiments, citric acid (Sigma) was added to 7H9 medium to yield pH values of 4.2 and 5.5. Control cultures contained no menadione or citric acid respectively. Cultures were incubated at 37°C with shaking. For studies with menadione, optical densities were measured over 13 days, aliquots from some time points were plated on 7H0 agar, and cfu were enumerated. For studies with citric acid, cfu were determined at the initiation of culture and at days 1 and 4.

RNA isolation

To isolate RNA from mycobacteria cultured in 7H9 medium, RNA was extracted using RNAzol B (TEL-TEST), according to the manufacture's instructions, except that lysing matrix B and a FastPrep FP120 shaker (both from BIO 101) were used to disrupt the mycobacteria. Chromosomal DNA was removed with DNA-free (Ambion) according to the manufacturer's instructions.

To isolate RNA from intracellular mycobacteria, the macrophages or MonoMac6 cells were lysed with 1 ml of extraction buffer (2 M guanidine isothiocyanate, 200 mM Tris-HCl, pH 8.3, 300 mM LiCl, 10 mM EDTA) per 10^6 cells. After sonication for 10 s with an ultrasonic cell disrupter (Virsonic 50; Virtis), the suspension was centrifuged at 7000 *g* for 10 min to pellet the bacilli. The pellet was washed with the extraction buffer, and RNA was extracted as outlined above for mycobacteria cultured in 7H9 medium.

Relative quantification of mRNA by real-time PCR

A mixture of 37 genome-directed primers designed to prime all open reading frames in the M. tuberculosis genome was used for reverse transcription (Talaat *et al*., 2000). Reaction mixtures of 20 ml included 500 ng of RNA denatured for 5 min at 65°C, 0.5 mM each dNTP, 1.0 mg of a mixture of 37 genome-directed primers, 10 U of RNase inhibitor (Promega), 2 ml of 10× buffer RT (Qiagen) and 4 U of Omniscript reverse transcriptase (Qiagen). Reactions were performed at 37°C for 60 min, followed by heating to 93°C for 5 min to inactivate the reverse transcriptase.

cDNA was quantified by real-time PCR, using the ABI Prism 7700 (Applied Biosystems). The sequences of the primers and probes are shown in Table 1. PCR conditions were identical for all reactions, which were performed in triplicate. Each 25 ml reaction contained 1× TaqMan universal PCR master mix (Applied Biosystems), 0.3 mM each primer, 0.2 mM probe and 10 ng of cDNA or DNA as template. Forty cycles of amplification were used, each consisting of denaturation at 95°C for 15 s, followed by annealing and extension at 60°C for 60 s. Standard curves for all target genes were generated by amplification of serial 10-fold dilutions of 10 ng ml^{-1} genomic DNA, using the appropriate primers and probes. Using the standard curve for each gene, the relative amount of cDNA in each sample was determined. To normalize for differences in efficiency of RNA extraction and reverse transcription, reactions for target genes and 16S rRNA were run simultaneously, and the amount of target cDNA was normalized for the amount of 16S rRNA present in that sample.

Table 1. Primers and probes used to measure mRNA expression by real-time PCR.[a]

Gene	Forward primer	Reverse primer	Probe[b]
sigA	gtgacccgggaacgcat	gtgagcggctcggatgg	cgccagatcgaatccaagactatgtcga
sigB	gcgcttcggcctgga	gcccgaatagtttgccgatt	cggccaaccacgcaccctg
mce1	cgggctgggcgataagtt	gcgaattgaggtcatccagaa	cgagtcgatcgtcaacgccaaca
16S rRNA	tcccgggccttgtacaca	ccactggcttcgggtgtta	cgcccgtcacgtcatgaaagtcg
23S rRNA	tgggctgttcgcccatta	gcaactgtctcacgacgttctaa	cccagctcgcgtgccgct

a. All primer and probe sequences are written in 5′ to 3′ order.
b. All probes were labelled with 5′-fluorescein phosphoramidite and 3′-TAMRA.

To use real-time PCR to compare *sigA* mRNA expression by the *M. tuberculosis* 210 strain isolate TB294 and H37Rv, we first confirmed that the strains had no mutations in the target sequences that altered the efficiency of PCR amplification. We used probe and primer sets to amplify serial 10- to 1000-fold dilutions of known amounts of genomic DNA from H37Rv and TB294. The cycle thresholds for amplification of DNA encoding *sigA* and 16S rRNA were identical for equal amounts of genomic DNA for both strains, demonstrating that the primers and probes have equivalent efficiency to detect these RNAs of the two strains. All sets of primers and probes yielded no PCR product when cDNA from uninfected macrophages was used as template.

Construction of sense and antisense sigA *transformants*

Genomic DNA was prepared from TB294 using the Wizard Genomic DNA purification kit (Promega). Standard DNA cloning procedures were followed (Sambrook *et al*., 1989). All transformations were performed with the *E. coli* NovaBlue strain (Novagen), which was grown in Luria–Bertani broth or on Luria–Bertani agar (Difco) at 37°C. The full-length *sigA* gene (Rv2703) was cloned from TB294 chromosomal DNA by PCR, using the forward and reverse primers, 5′-gtggcagc gaccaaagca-3′ and 5′-ccaggtagtcgcgca-3′, respectively, to which *Eco*RI and *Cla*I tags were added as appropriate. The *sigA* gene was then inserted in the forward (sense) or reverse (antisense) direction downstream of the hsp60 promoter in the *E. coli–M. tuberculosis* shuttle vector pMV261 (Stover *et al*., 1991) to make sense and antisense *sigA* constructs. We confirmed that these constructs contained full-length *sigA* in the appropriate orientation by restriction enzyme digestion, followed by sequencing. The plasmids, including the vector control, were then electroporated into TB294 and H37Rv. For the sense and antisense *M. tuberculosis* transformants, the presence of the kanamycin resistance gene was confirmed by PCR. Plasmids were also isolated, and restriction enzyme digestion confirmed the presence of *sigA*.

Western blotting

MonoMac6 cells were infected with TB294 and H37Rv transformants at an MOI of 1:1. After 6 days, the cells were lysed with extraction buffer (2 M guanidine isothiocyanate, 200 mM Tris-HCl, pH 8.3, 300 mM LiCl, 10 mM EDTA) per 10^6 cells for 5 min, then vortexed for 1 min. The suspension was centrifuged at 7000 *g* for 10 min. The pellet was resuspended and protein was extracted, using the B-PER kit (Pierce), according to the manufacturer's instructions, except for the additional step of disrupting the mycobacteria with a Fast-Prep FP120 shaker and lysing matrix B. The B-PER kit was also used to extract proteins from *M. tuberculosis*, *M. smegmatis* and *E. coli* cultured in broth and from uninfected MonoMac6 cells. The additional disruption step was omitted for extraction of proteins from *E. coli* and uninfected MonoMac6 cells. The amount of protein in each sample was quantified by the bicinchoninic acid method (Pierce).

Using 20–50 mg of protein extract in each sample, 10% SDS-PAGE was performed under reducing conditions by standard methods (Laemmli, 1970), and Western blotting was performed by standard methods, using a 1:2000 dilution of the mAb 2G10 (Strickland *et al*., 1988; Predich *et al*., 1995) (Neoclone Biotechnology) and a 1:8000 dilution of secondary Ab (rabbit anti-mouse IgG conjugated to horseradish peroxidase; Santa Cruz Biotechnology). Ab binding was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). To detect total protein on the SDS polyacrylamide gel, Coomassie brilliant blue R-250 staining and destaining solutions were used, according to the manufacturer's instructions (Bio-Rad).

Infection of mice with sense and antisense sigA *transformants*

We used 8- to 10-week-old C57Bl/6 mice that had been maintained in specific pathogen-free conditions. The TB294 and H37Rv transformants were grown to mid-log phase in glycerine alanine salts medium containing 0.01% Tween 80 and stored in ampoules at −70°C until use. An aerosol generator (Glas-Col) was used to deposit ≈100 bacteria in the lungs of each mouse. The number of viable bacteria in the lungs was determined at days 1, 10 and 20 by plating serial dilutions of partial lung homogenates from four to seven mice on Middlebrook 7H11 agar and counting colonies after 21 days. The posterior lobe of the right lung was collected at day 20, fixed overnight in 10% (v/v) phosphate-buffered formalin, embedded in paraffin and processed for histological examination.

Statistical analysis

Statistical comparisons for data that were normally distributed were made by the Student's *t*-test. Data that were not normally distributed were compared by the Wilcoxon rank-sum test. Values were expressed as mean ± standard error.

For comparison of cfu of TB294 transformants in murine lungs, two-way ANOVA was used.

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