Examination of *Mycobacterium tuberculosis* sigma factor mutants using low-dose aerosol infection of guinea pigs suggests a role for SigC in pathogenesis

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Secondary sigma factors in bacteria direct transcription of defence regulons in response to specific stresses. To identify which sigma factors in the human respiratory pathogen *Mycobacterium tuberculosis* are important for adaptive survival *in vivo*, defined null mutations were created in individual sigma factor genes. In this study, *in vitro* growth virulence and guinea pig pathology of *M. tuberculosis* mutants lacking functional sigma factors (SigC, SigF, or SigM) were compared to the parent strain, H37Rv. None of the mutant strains exhibited a growth deficiency in Middlebrook 7H9 broth, nor were any impaired for intracellular replication in the human monocytic macrophage cell-line THP-1. Following low-dose aerosol infection of guinea pigs, however, differences could be detected. While a SigM mutant resulted in lung and spleen granulomas of comparable composition to those found in H37Rv-infected animals, a SigF mutant was partially attenuated, exhibiting necrotic spleen granulomas and ill-defined lung granulomas. SigC mutants exhibited attenuation in the lung and spleen; notably, necrotic granulomas were absent. These data suggest that while SigF may be important for survival in the lung, SigC is likely a key regulator of pathogenesis and adaptive survival in the lung and spleen. Understanding how SigC mediates survival in the host should prove useful in the development of anti-tuberculosis therapies.

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INTRODUCTION

Tuberculosis (TB) remains a major health problem in most parts of the world. Despite decades of effort to eliminate the disease, *Mycobacterium tuberculosis* infection remains the leading cause of death and disease by a single bacterial human pathogen, claiming approximately two million lives each year (World Health Organization, 2005; Dye *et al.*, 1999). Although the *M. bovis* BCG vaccine is widely used to prevent mycobacterial meningitis in children, its efficacy against pulmonary TB is highly variable (0–80 %; World Health Organization, 1979; Rodrigues & Smith, 1990).

Understanding how *M. tuberculosis* bacilli adapt and survive upon encountering hostile environments inside the host is critical to producing effective vaccines and therapies. Bacteria employ multiple secondary sigma factors that sense specific stress signals and transiently coordinate expression of genes encoding functions that facilitate bacterial adaptation to those particular stresses. Sequence analysis of the *M. tuberculosis* strain H37Rv suggested the presence of 12 secondary sigma factor genes (*sigB*–*sigM*) and a primary sigma factor gene, *sigA* (Cole *et al.*, 1998).

Studies of knockout mutations of secondary sigma factor genes in *M. tuberculosis* strains suggest some sigma factors play roles in virulence. While the *sigD* and *sigF* genes are apparently not required for survival in macrophage infections, a *sigD* mutant of H37Rv and a *sigF* mutant of

Abbreviations: AFB, acid-fast bacilli; TB, tuberculosis.

strain CDC1551 were each attenuated for pathogenesis in BALB/c mice (Raman *et al.*, 2004; Chen *et al.*, 2000). The *sigF* mutant was subsequently shown to exhibit reduced persistence in the lungs and spleen of BALB/c mice (Geiman *et al.*, 2004). A *sigH* mutant of CDC1551 survived in macrophages and persisted in the lungs and spleen of C57BL/6 mice, suggesting that SigH had no role *in vivo*. However, in C3H/HeJ mice, SigH contributed to tissue damage and death of the host (Kaushal *et al.*, 2002). A *sigC* mutant of CDC1551 was reported to be less virulent, but just as persistent as the parental strain in DBA/2 mice (Sun *et al.*, 2004). SigI appeared not to be required for survival in BALB/c mice (Hu *et al.*, 2004). SigE, however, was reported to be required for survival of H37Rv in macrophages (Manganelli *et al.*, 2001). In addition, a *sigE* mutant persisted less effectively than the H37Rv parent strain in the lungs, spleen and liver of BALB/c mice, and mice infected with the mutant survived longer than those infected with the parent strain (Manganelli *et al.*, 2004). Interestingly, while C3H/HeJ mice infected with a *sigE* mutant of the CDC1551 strain also survived longer than those infected with the parent strain, the mutant was not impaired for lung persistence (Ando *et al.*, 2003). Genetic differences between the parent strains of the *sigE* mutants or differential susceptibilities in the murine models were posited to explain the observed disparity in persistence (Manganelli *et al.*, 2004). Such phenotypic variability highlights the importance of examining mutants in different genetic backgrounds and the need for study in multiple animal model systems.

While murine models of TB have the advantages of low cost, availability of immunological reagents, and the choice of inbred populations with varying susceptibility to aerosol infection, guinea pigs are considered a superior model of human *M. tuberculosis* infection in terms of lung granuloma formation and function, subsequent necrosis, caseation and disease progression (Baldwin *et al.*, 1998; Turner *et al.*, 2003; McMurray *et al.*, 1996; Orme *et al.*, 2001; Ridley & Ridley, 1987). The classical human lung granuloma contains a caseous necrotic core with varying degrees of calcification surrounded by epithelioid macrophages and layers of T lymphocytes and fibrous proteins (Cotran *et al.*, 1999). While these features are evident in the guinea pig TB model, the same does not hold for murine models. Murine granulomas are structured differently, containing significant numbers of B lymphocytes (Gonzalez-Juarrero *et al.*, 2001). Mice also produce an unusually strong cellular immune response to *M. tuberculosis* bacilli that limits lung damage and enables sustained bacillary loads in excess of 10^6 per lung for many weeks (Orme *et al.*, 2001). In addition, it may take months, if ever, for murine granulomas to exhibit central necrosis, but calcification never occurs (Rhoades *et al.*, 1997).

In this study, we employed low-dose aerosol infection of guinea pigs for screening sigma factor knockout mutants of strain H37Rv to identify sigma factors that may be important regulators of virulence. Mutants defective in production of SigC produced the most-attenuated phenotypes,

exhibiting limited pathogenesis in the lungs and spleen. This work suggests that SigC has a role in granuloma formation in the lungs and in successful dissemination and establishment of infection in other organs.

METHODS

Enzymes and reagents. High-fidelity *Pfu* DNA polymerase was purchased from Stratagene. Restriction endonucleases, DNA-modifying enzymes, nucleotides and DNA standards were purchased from New England Biolabs. Antibiotics were purchased from Research Products International. Primers were synthesized by the Centers for Disease Control and Prevention (CDC) core facility or purchased from Integrated DNA Technologies. Primers used in these studies are indicated in Table 1.

Bacterial strains. All *M. tuberculosis* mutant strains in this report were derived from virulent strain H37Rv obtained from the CDC mycobacterial strain collection. Mycobacterial strains were cultured in Dubos medium supplemented with OADC (BD/Difco) and 0.05% Tween 80. Replicate bacterial stocks used for infections were prepared from cultures grown to exponential phase (OD_{580} 0.5, measured with a Thermo-Spectronic 20D + spectrophotometer). Bacterial titres were obtained from thawed stocks after serial dilution in culture medium and plating on Middlebrook 7H11 agar with OADC supplementation (BD/Difco). When appropriate, the antibiotic kanamycin or hygromycin was used at 25 and 50 $\mu\text{g ml}^{-1}$, respectively.

Plasmid constructions. Plasmid pPR27 Δ sigMhyg, used to create a *M. tuberculosis* *sigM* deletion mutant (*sigMhyg*) marked with a hygromycin resistance gene from p16R1, *hyg*, was made as follows. The *sigM* gene + ~500 bp flanking regions obtained by PCR with *M. tuberculosis* DNA template, *Pfu* DNA polymerase, dNTPs and primers sigMF1/sigMR1 was cloned into plasmid pCR2.1TOPO (Invitrogen). An internal deletion of *sigM* was obtained by digesting the resulting plasmid with *Sac*II and *Age*I, blunting the DNA ends with T4 DNA polymerase, and ligating with the blunted ~1300 bp *Bsp*HI–*Sma*I *hyg* gene from p16R1 (Yuan *et al.*, 1998) to form

Table 1. Primers

Primer	Sequence (5'–3')
sigCF1	GGGATCCTGAACGTGTTGTTGGGCCA
sigCR1	GGGATCCGGTACCGCCA
sigMF1	GGACTAGTCTTGGCTTCGGTGTTGA
sigMR1	GGACTAGTGTGGTGAAGCAGGTCGA
usFXF1	ATGGATCCGGTGCTGACGCTCTGCATAG
sigFR5	GAGGATCCTCGGTACATACCCGCTGG
sigFAF1	CCTTAATTAACGTCGGCGTATTCTGTTAGCT
sigFAR1	CCTTAATTAAGTCATTGGCAGCGGCTACGG
up565sigC	GGGGTACCGTCGCTATAGCGTATC
down92sigC	CGCAAGCTTCGGTGGTCATGATAGC
sigMoutF1	GAGTCAAGCCGACATCTCG
sigMoutR1	GATCGATGCTACGACGGAC
sigFoutF1	CATCGCGAGCTAACGAATAC
sigFoutR1	CGTAGCCGTGCCAATGACT
sigCoutF1	CACCCAAGAGACATTCTAC
sigCoutR1	TTACCACGATGAGTTCGCAC
sigC10F	ACGGCAAGCGACGACGAG
sigC379R	TCGTGGTTACCTCGACGAG

plasmid pCR2.1 Δ sigMhyg. The *SpeI* fragment containing the Δ sigMhyg + ~500 bp flanking regions was then cloned into plasmid pPR27 (Pelicic *et al.*, 1997) to form plasmid pPR27 Δ sigMhyg.

Plasmid pPR27 Δ sigFhyg, used in the creation of a *hyg*-marked *sigF* deletion mutant in *M. tuberculosis* (*sigFhyg*), was produced as follows. The *sigF* gene plus flanking regions (1140 bp upstream, 500 bp downstream), obtained by PCR from *M. tuberculosis* template with primers usXF1/sigFR5, was TA-cloned into plasmid pCR2.1TOPO, creating plasmid pRKK413. The *sigF* gene was deleted from this plasmid by PCR using primers sigF Δ F1/sigFAR1 to form plasmid pCR2.1 Δ sigF1. The *hyg* gene was inserted into the *PacI* site of pRKK413 to create pCR2.1 Δ sigFhyg. The *Bam*HI fragment containing Δ sigFhyg plus flanking sequences was then ligated into the *Bam*HI site of pPR27 to form pPR27 Δ sigFhyg.

Plasmid pPR27sigChyg, used in generating a *hyg*-marked disruption mutation in the *SigC*-coding region of *M. tuberculosis* (*sigChyg*), was constructed as follows. A DNA segment carrying *sigC* plus ~500 bp flanking regions obtained from *M. tuberculosis* template DNA (by PCR with primers sigCF1 and sigCR1) was TA-cloned into vector pCR2.1TOPO to form plasmid pCR2.1sigC. A 1293 bp hygromycin resistance gene, *hyg*, obtained as a *SmaI*-*Bsp*HI fragment from plasmid p16R1, was inserted into the unique *Eco*NI site within *sigC* to form plasmid pCR2.1sigChyg. The *Bam*HI fragment encoding the *hyg*-disrupted *sigC* gene plus ~500 bp flanking regions from this plasmid was ligated into plasmid pPR27 to form pPR27sigChyg.

Plasmid pLJ Δ sigC used in the creation of an unmarked internal *sigC* deletion mutant of *M. tuberculosis* (*Rv-sigC*) was constructed as follows. The 1569 bp *Bam*HI fragment containing *sigC* plus ~500 bp flanking regions was excised from pCR2.1sigC and inserted into the *Bam*HI site of plasmid pUC19spF' (Erickson & Gross, 1989). An internal deletion of the carboxy-terminal third of *sigC* was obtained by removing the sequence between the *Eco*NI and *Xho*I sites to form pUC19spF Δ sigC. The *Bam*HI fragment containing Δ sigC plus 500 bp flanking sequences was excised from this plasmid and ligated into the *Bam*HI site of plasmid pLCG2 to form plasmid pLCG2 Δ sigC. Plasmid pLCG2 is a derivative of p2NIL (Parish & Stoker, 2000) in which the *PacI* site was destroyed by insertion of a *PmeI* linker (New England Biolabs). The *PacI* fragment encoding the *lac-sac* region from pGOAL17 was then inserted into the *PmeI* site of pLCG2 Δ sigC to create pLJ Δ sigC.

Complementation plasmid pMV306sigC was created as follows. The *M. tuberculosis* *sigC* region (containing 565 bp of upstream regulatory sequence to 92 bp downstream of *sigC*), obtained by PCR using primers up565sigC/down92sigC, was ligated into the *Hind*III site of the mycobacterial integrating vector pMV306 (Kong & Kunitomo, 1995).

Isolation of *M. tuberculosis* mutants. The hygromycin-marked *M. tuberculosis* sigma factor knockout strains (*sigChyg*, *sigFhyg*, *sigMhyg*) were obtained using the homologous recombination method of Pelicic *et al.* (1997) following electroporation of H37Rv with a plasmid derived from the mycobacterial temperature-sensitive replicating plasmid pPR27 (pPR27sigChyg, pPR27 Δ sigFhyg, pPR27 Δ sigMhyg, respectively). The unmarked *sigC* mutant (*Rv-sigC*) was obtained following electroporation of H37Rv with the suicide plasmid pLJ Δ sigC using the unmarked deletion method (Parish & Stoker, 2000). Strain *sigChyg* was complemented with a wild-type copy of the *sigC* gene by introduction of plasmid pMV306sigC, resulting in strain *sigComp*. All plasmids were sequenced to check for the absence of PCR-generated mutations.

THP-1 infections. The human monocytic cell line THP-1 was cultured in RPMI medium supplemented with 10% fetal bovine serum (RPMI + 10% FBS). For infection assays, 10⁶ monocytes were seeded into each well of 24-well dishes. Differentiation into

macrophages was achieved by incubation for 3 days following addition of 100 nM phorbol esters (Sigma-Aldrich). The macrophage monolayers were incubated for 24 h in medium lacking phorbol esters prior to infection. Cells were infected for 6 h with the indicated *M. tuberculosis* strains at an m.o.i. of approximately 10 bacilli per host cell by addition of identical volumes from bacterial stocks previously stocked at identical cell densities (OD₅₈₀ = 0.5). Non-adherent bacilli were removed by washing the monolayers with Hank's Balanced Salts Solution (HBSS, Invitrogen). External bacilli not removed by washing were prevented from replicating by 2 h incubation of the monolayers in RPMI + 10% FBS containing 200 μ g amikacin ml⁻¹. The monolayers were again washed with HBSS and then covered with 1 ml RPMI + 10% FBS. At the indicated timepoints, THP-1 cells were lysed by the addition of Triton X-100 to 0.1%. Viable bacilli were enumerated by serial dilution in Middlebrook 7H9 medium containing 0.05% Tween 80 and supplemented with 10% ADC (BD/Difco) and plating on Middlebrook 7H11 agar supplemented with 10% OADC (BD/Difco) and 0.05% Tween 80. Over the 7 day infection period, uninfected THP-1 monolayers progressed from 50% to almost complete confluence. Over a comparable period, infected monolayers remained at approximately 50% confluence although an increased number of detached cells were detected as the infections proceeded.

Animal infections. Female outbred Hartley guinea pigs (~500 g) used in this study were purchased from the Charles River Laboratories. *M. tuberculosis* H37Rv and mutant derivatives were grown to exponential phase in Dubos medium containing 0.05% Tween 80 and supplemented with 10% Middlebrook OADC (BD/Difco). Cultures were divided into 1 ml aliquots and frozen at -80 °C until used. Thawed aliquots were diluted in double-distilled sterile water to the desired inoculum concentration. Animals were infected using a Madison aerosol generation chamber (Wiegand *et al.*, 1970) calibrated to deliver approximately 30 c.f.u. *M. tuberculosis* bacilli into the lungs of each guinea pig. Except as otherwise indicated, four animals infected with each strain were sacrificed at each timepoint. The lower left lung lobe and distal third of the spleen from each animal were fixed in 10% formalin for 20 h and then placed in 70% ethanol. Tissues with the most pathology for each specimen were paraffin embedded and 4 μ m tissue sections were stained with haematoxylin and eosin for histopathology and Ziehl-Neelsen stains for acid-fast bacilli (AFB).

RESULTS

Creation of sigma factor mutants

We initially examined three sigma factor null strains: *sigChyg*, *sigFhyg* and *sigMhyg*. The mutant strains were created by insertion of a hygromycin resistance gene (*hyg*) within the coding region of the specified sigma factor gene (*sigC*, *sigF* and *sigM*, respectively) in *M. tuberculosis* strain H37Rv. The orientation of the *hyg* gene relative to the sigma factor coding sequence and to adjacent genes on the chromosome is shown in Fig. 1. Mutants were identified by PCR and confirmed by Southern analysis (data not shown).

Examination of *in vitro* growth characteristics and intracellular survival of sigma factor mutants

General growth characteristics of the hygromycin-marked sigma factor mutant strains (*sigChyg*, *sigFhyg* and *sigMhyg*) and the H37Rv parent strain were monitored.

Fig. 1. (A, B, C) Sigma factor mutations and PCR analysis. Sigma factor genes *sigM*, *sigF* and *sigC* are drawn with putative adjacent chromosomal ORFs based on information from the Tuberculist database. ORFs (boxes) are approximately to scale relative to each other. ORFs or multiple ORFs extending beyond the figure are indicated (jagged-ended boxes). The orientation of each ORF is indicated (arrows). Insertion sites of the hygromycin resistance gene, *hyg*, present in each of the marked sigma factor mutants (*sigMhyg*, *sigFhyg*, *sigChyg*) are indicated above each sigma factor gene (dashed lines). The internal region of *sigC* deleted in the unmarked *sigC* mutant strain *Rv-sigC* is indicated by the shaded region within the Δ *sigC* box located below the *sigC* gene (C). (D) The *sigC* gene and flanking regions (boxes) cloned into the integrating plasmid pMV306 (thick circular line) used for complementation studies are indicated.

Mid-exponential-phase cultures ($OD_{580} = 0.5$) were used to inoculate 250 ml capped side-arm flasks containing 50 ml Middlebrook 7H9 plus ADC enrichment to an OD_{580} of 0.01 unit. Similar growth kinetics was observed among all of these shaking cultures, indicating that the mutants were not impaired for growth under these conditions (data not shown). Because survival in human macrophages is essential for *M. tuberculosis* disease progression, the mutants were next examined for the ability to replicate inside the human monocytic macrophage cell line THP-1 (Fig. 2). Initial differences at the $t=0$ timepoint may reflect differences in either self-adherence or adherence of the different strains to host cells following introduction into the cell culture medium as it lacks the anti-clumping non-ionic detergent Tween 80. However, each of the sigma factor knockout strains achieved similar titres by day 6 post-infection, suggesting that none are impaired in intracellular replication.

Examination of guinea pig lung infection and dissemination to the spleen

To determine which of the sigma factor mutants is required for adaptive survival upon entering a mammalian host,

Fig. 2. Replication of *M. tuberculosis* strains in THP-1 macrophages. THP-1 macrophages were infected with the indicated strains at an m.o.i. of 10 for 6 h, followed by 2 h incubation with amikacin ($200 \mu\text{g ml}^{-1}$) to prevent replication of external bacilli. After washing with HBSS, fresh medium was placed on the monolayers. Bacteria were enumerated at the indicated timepoints following lysis of monolayers with 0.1% Triton X-100, serial dilution in medium containing 0.05% Tween 80 and plating on Middlebrook 7H11 medium. Data presented are the mean of two experiments performed in triplicate. Error bars indicate standard deviation.

each was introduced into guinea pigs via the respiratory route using a Madison Chamber calibrated to deliver approximately 30 c.f.u. to the lungs. The infecting bacilli were previously grown to early exponential phase growth ($OD_{580} = 0.5$) in rich medium to minimize expression of individual stress-response sigma factor genes prior to infection. Animals were monitored over a 20 week period. The three marked sigma factor mutant strains *sigMhyg*, *sigFhyg* and *sigChyg* were initially compared against the parental strain H37Rv. Subsequently, two additional *sigC* mutant strains were tested. Strain *sigCcomp* complemented the marked *sigChyg* strain with a wild-type copy of *sigC* integrated at the mycobacteriophage L5 attachment site on the chromosome. Strain *Rv-sigC* contains an unmarked internal deletion within *sigC* to control for the possibility that the *sigChyg* phenotypes were not caused by increased expression of *blaC* due to read-through from the *hyg* gene (see Fig. 1).

Histopathological analyses of the lungs and spleen were performed. In general for each strain, the granulomas in the lung and spleen at early timepoints (5–6 weeks post-infection) appeared to be larger (some having necrotic centres), while fewer granulomas with reduced pathology were observed at 20 weeks post-infection. The *sigC* mutants produced the least pathology of all of the strains examined for granuloma formation, necrosis and mineralization (Figs 3, 4 and 5, Table 2). While medium and large granulomas were detected in the lungs of animals at both

Fig. 3. Photomicrographs of histopathological preparations of lungs infected with the different mutants at 5–6 weeks post-infection. Medium and extra large, well-formed granulomas (G) are evident in guinea pigs infected with H37Rv and *sigMhyg*, respectively. The granuloma formed with *sigMhyg* also showed a central necrotic (N) core. A small, well-formed granuloma was found with *sigChyg*. The *sigFhyg* strain resulted in ill-defined granulomas. Bronchioles (B) and prominent blood vessels (V) are labelled for reference. (Haematoxylin and eosin stains, original magnification 25 ×.)

early and late times following infection with strains carrying a wild-type copy of *sigC* (H37Rv, *sigMhyg*, *sigFhyg*, *sigCcomp*), only a single small granuloma was detected in an animal 6 weeks post-infection with one of the *sigC* mutants (*sigChyg*). The granulomas from the *sigFhyg*-infected animals appeared less-defined in the lung compared to the spleen at early and late timepoints (Figs 3, 4 and 5, Table 2). Granulomas with detectable AFB were observed in the spleen of animals at 5–6 weeks following infection with H37Rv, *sigMhyg*, *sigFhyg*, but not with either *sigC* knockout mutant or, interestingly, the complemented strain (*sigCcomp*, Table 2). At 20 weeks post-infection, granulomas with detectable AFB were observed in the spleen of animals infected with either H37Rv or *sigFhyg*, suggesting that these granulomas were induced by the presence of *M. tuberculosis* bacilli (Table 2). While a granuloma was detected in the spleen from one animal infected with *Rv-sigC* at 20 weeks post-infection, no associated AFB were found. Therefore, we can not formally conclude that this granuloma was induced by mycobacteria.

Necrosis within granulomas was evident in the lungs of animals 5–6 weeks post-infection with H37Rv or *sigMhyg*, and in spleens infected with H37Rv, *sigMhyg* or *sigFhyg* (Table 2, Figs 3, 4 and 5). While a granuloma was detected in the lung of an animal infected with the complemented *sigC* mutant strain (*sigCcomp*), the absence of necrosis was unexpected. This may have been due to aberrant expression of a wild-type copy of *sigC* integrated at the L5 mycobacteriophage attachment site on the chromosome. Calcification of the necrotic centres was evident in lung granulomas of animals infected with either H37Rv or *sigMhyg* at 20 weeks post-infection (Table 2, Fig. 5). The lack of necrosis in granulomas from any animal infected with either *sigC* knockout (*sigChyg* or *Rv-sigC*) suggests that SigC regulates factors important for host immune modulation.

DISCUSSION

In this study, *M. tuberculosis* mutants of *sigC*, *sigF* and *sigM* were created in strain H37Rv and compared with one

Fig. 4. Photomicrographs of histopathological preparations of spleens infected with the different mutants at 5–6 weeks post-infection. Large, well-formed granulomas (G) with necrotic (N) core are noted for guinea pigs infected with H37Rv, *sigFhyg*, or *sigMhyg*. Spleen tissue from the *sigChyg*-infected animals appeared relatively normal, consisting of erythrocyte-enriched sinusoids [also known as red pulp (R)] and lymphocyte-enriched white pulp (W). (Haematoxylin and eosin stains, original magnification 25 ×.)

another for alterations in growth and virulence phenotypes *in vitro* and in the guinea pig model of TB pathology. The lack of growth differences in broth cultures among the mutants and parent strain indicates that none of these sigma factors is critical for survival under non-stressed conditions. This is consistent with transposon site hybridization analyses that previously indicated that the genes encoding these sigma factors were non-essential for *in vitro* growth (Sassetti *et al.*, 2003). The ability of all the mutants to replicate inside THP-1 macrophages suggests that none of these sigma factors exclusively regulate genes that are critical for survival in macrophages. The ability of the *sigC* and *sigF* mutants of strain H37Rv to replicate in macrophages by *sigC* and *sigF* mutants derived from *M. tuberculosis* strain CDC1551 (Sun *et al.*, 2004; Chen *et al.*, 2000).

The goal of this work was to use the guinea pig model as a screen for *M. tuberculosis* sigma factor mutants to identify

those that mediate adaptive survival and pathogenesis upon entering the host environment. Although different murine strains have been used to model the relevance of specific sigma factors to human TB disease, this is believed to be the first reported use of the guinea pig model to examine the contributions of sigma factors to *M. tuberculosis* virulence. To mimic the typical route of human infection, the animals were infected via low-dose aerosols that deliver approximately 30 c.f.u. of bacilli to the lungs. Reflecting the outbred nature of guinea pigs, variability in tissue pathology was evident among replicate animals infected with the same bacterial strain (Table 2). However, general differences in granuloma size and composition were detectable among the sigma factor mutants. A *sigM* mutant was the least attenuated. It resulted in necrotic granulomas in the lungs and spleen much like those observed in H37Rv-infected animals. In addition, as with H37Rv-infected animals, calcification of lung granulomas was evident at 20 weeks post-infection. Thus, it would seem that SigM is not essential for virulence in this host.

Fig. 5. Photomicrographs of histopathological preparations of lungs infected with the different mutants at 20 weeks post-infection. Large, well-formed granulomas (G) are noted for guinea pigs infected with H37Rv or *sigMhyg*. The granuloma formed with the *sigMhyg* mutant also showed a central necrotic and calcified (C) core. The guinea pig infected with the *sigFhyg* mutant contained a small, non-necrotic granuloma (G). Relatively normal 'lacelike' appearance of healthy lung tissue due to the absence of alveolar airsac staining is observed from animals infected with the *sigChyg* mutant. Bronchi (B) and blood vessels (V) are also indicated. (Haematoxylin and eosin stains, original magnification 25 ×.)

A *sigF* mutant resulted in diffuse guinea pig lung granulomas lacking necrosis, but surprisingly normal-appearing granulomas with necrotic centres in the spleen. The lung phenotype is generally consistent with a murine study of a *sigF* mutant of CDC1551 which reported reduced lung granulomatous inflammation relative to the parent strain between 12 and 20 weeks post-infection (Geiman *et al.*, 2004). The presence of necrotic guinea pig spleen granulomas containing AFB suggests that differences in the internal environments of these organs affect the survival of SigF-deficient bacilli. Geiman *et al.* (2004) reported a reduction in envelope-associated sulfolipids in a *sigF* mutant. Reduced amounts of this or other SigF-regulated products may result in heightened susceptibility to oxidizing agents or surfactants found exclusively in the lungs of guinea pigs and mice.

Studies of the three *M. tuberculosis* sigma factor mutants in guinea pigs suggest that SigC is more important for

pathogenesis and survival within granulomas than either SigF or SigM. SigC-deficient bacilli resulted in fewer and smaller lung and spleen granulomas. In addition, necrotic centres were absent from granulomas in the lung of animals infected with a *sigC* mutant. This suggests that SigC is an important regulator of virulence. This view is consistent with observations that a *sigC* mutant of a recent clinical *M. tuberculosis* strain (CDC1551) produced fewer granulomas in DBA2 mice (Sun *et al.*, 2004). However, a difference in persistence of *sigC* mutants between these models may exist. In this study, the lack of lung granulomas or detectable AFB suggests that *sigC* mutants derived from strain H37Rv do not persist to 20 weeks post-infection. This contrasts with the report that CDC1551 *sigC* mutant bacilli maintained elevated lung titres for a comparable period (Sun *et al.*, 2004). This difference in persistence may be due to inherent differences between H37Rv and CDC1551. Alternatively, physiological differences between these animal models might also explain

Table 2. Histopathological analysis of guinea pig organs following infection with *M. tuberculosis* sigma factor mutants

Pathological assessments were made blinded to the identities of the infecting strains (left column). Equivalent amounts of tissue were examined from each animal.

Strain	Time (weeks)	No. of pigs tested	Lungs				Spleen			
			No. of pigs with granulomas	Granulomas and size*	Granulomas with necrosis	Granulomas with AFB (no. of AFB)	No. of pigs with granulomas	Granulomas and size†	Granulomas with necrosis	Granulomas with AFB (no. of AFB)
H37Rv	6	4	4	4 MED, 4 SM	1	1 (2)	2	>3 LG, 2 MED	1	2 (3–4)
<i>sigMhyg</i>	6	4	4	>3 LG, >7 MED	2	2 (4–10)	1	>3 LG	1	1 (4–6)
<i>sigFhyg</i>	6	4	3	2 MED‡, 1 SM‡	0	0	2	2 LG, 1 MED	1	2 (1–2)
<i>sigChyg</i>	6	4	1	1 SM	0	0	0	0	0	0
H37Rv	17	4	3	>3 LG, 3 SM	1	0	0	0	0	0
<i>sigMhyg</i>	17	4	2	>6 XLG	1	3 (4–10)	3	>3 XLG, >3 MED	1	1 (>10)
<i>sigFhyg</i>	17	4	0	0	0	0	1	5 MED	0	1 (1)
<i>sigChyg</i>	17	4	0	0	0	0	1	2 SM	0	0
H37Rv	20	2	2	4 LG	2 (calcified)	0	1	3 LG	1	1 (4–10)
<i>sigMhyg</i>	20	2	1	1 LG	1 (calcified)	0	0	0	0	0
<i>sigFhyg</i>	20	2	1	1 SM	0	0	1	1 XLG	1	1 (4–10)
<i>sigChyg</i>	20	2	0	0	0	0	0	0	0	0
<i>Rv-sigC</i>	5	4	0	0	0	0	0	0	0	0
<i>sigCcomp</i>	5	4	2	3 MED, 1 SM	1	0	0	0	0	0
<i>Rv-sigC</i>	20	4	0	0	0	0	1	1 SM	0	0
<i>sigCcomp</i>	20	4	2	>5 MED, 2 SM	0	0	0	0	0	0

*The total number of granulomas from all animals within each group is indicated. The approximate sizes of granulomas are ranked from extra large (XL) to small (SM). The sizes of the granulomas were estimated as follows: SM, collection of epithelioid macrophages and lymphocytes without necrosis or calcification involving up to 6 alveoli; MED, collections involving up to 15 alveoli with or without necrosis; LG, coalescent collections with central necrosis but not larger than 30 alveoli; XLG, coalescent collections with central necrosis larger than 30 alveoli.

†Sizes based on scale used for lung granulomas. The numbers of granulomas with clearly defined central necrosis and those with subsequent calcification are indicated. The presence of AFB within granulomas is also indicated.

‡Denotes ill-defined granulomas.

the divergent survival. The ability of guinea pigs to produce granulomas with necrotic cores may result in effective killing of internalized *M. tuberculosis* compared to murine hosts, thereby minimizing dissemination.

The differences in guinea pig lung and spleen granulomas induced by *sigC* or *sigF* mutants suggest different mechanisms of resisting host defences. A *sigF* mutant maintains the ability to produce necrotic granulomas in the spleen, but produces ill-defined lung granulomas. In contrast, *sigC* mutants fail to cause the production of necrotic granulomas in either organ. Different promoter consensus sequences have been proposed for SigC and SigF, suggesting that each controls a unique regulon (Sun *et al.*, 2004; Geiman *et al.*, 2004). Given that each mutant produces limited lung pathology, it is possible that some genes may have promoters for both sigma factors. Expression of *sigC* was reported to be down-regulated in a *sigF* mutant in early stationary phase growth (Geiman *et al.*, 2004). The granuloma phenotypes are consistent with such a hierarchical regulation and suggest that SigC is more likely than SigF to be a direct regulator of genes that enable the bacilli to successfully adapt to critical host defences. Future efforts will seek to define the factors directly regulated by SigC, as they may serve as targets for vaccines or anti-mycobacterial therapies.

These studies support the hypothesis that SigC, and to a lesser extent SigF, is responsible for mediating adaptive survival of *M. tuberculosis* upon entering the host environment. It remains unclear what host signals activate expression or activity of these sigma factors. Further studies examining virulence of strains grown to stationary phase or taken from the surface pellicle of cells cultured under non-shaking conditions may result in bacilli expressing various stress response factors prior to entering the host. Bacilli pre-armed as such prior to infection would be expected to be more resistant upon initial encounter with host cells and may be more adept at redirecting the immune response away from a cell-mediated killing of this intracellular pathogen.

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