The alternative sigma factor SigF of *Mycobacterium smegmatis* is required for survival of heat shock, acidic pH and oxidative stress

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The alternative sigma factor SigF of *Mycobacterium tuberculosis* has been characterized in detail as a general-stress, stationary-phase sigma factor involved in the virulence of the bacterium. While a homologous gene has been annotated in the genome of the fast-growing *Mycobacterium smegmatis*, little experimental evidence is available on the function of this gene. Here, we demonstrate that SigF of *M. smegmatis* is required for resistance to hydrogen peroxide, heat shock and acidic pH, but not for survival in human neutrophils. No difference in sensitivity to isoniazid was observed between the wild-type strain and the $\Delta sigF$ mutant, suggesting that SigF-mediated resistance to hydrogen peroxide was via a pathway independent of KatG or AhpC. RT-PCR and 5'-RACE (rapid amplification of cDNA ends) analyses showed that *sigF* of *M. smegmatis* was co-transcribed with *rsbW* (thought to encode an anti-sigma factor for SigF) and MSMEG_1802 (unknown function) and was expressed from two promoters, one upstream of MSMEG_1802 and the second upstream of *rsbW*. Analysis of transcriptional *lacZ* fusion constructs in the *sigF*-deletion background revealed that the MSMEG_1802 promoter was dependent on SigF for expression. Moreover, MSMEG_1802-*lacZ* was induced twofold upon entry into stationary phase, while exposure of exponentially growing cultures to various stress conditions (e.g. heat, cold, ethanol, hydrogen peroxide or different pH values) did not lead to induction of MSMEG_1802-*lacZ*. Expression of *rsbW-lacZ* was independent of SigF and remained constant throughout the growth cycle and under various stress conditions unless the bacteria were challenged with D-cycloserine.

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INTRODUCTION

In the course of investigations into the molecular mechanisms underlying the pathogenesis and persistence of *Mycobacterium tuberculosis*, several studies have focused on the role of global regulators, such as sigma factors. The genome of *M. tuberculosis* encodes 13 sigma factors, two primary factors (SigA and SigB) and 11 alternative factors (SigC to SigM in alphabetical order) (Cole *et al.*, 1998; Manganelli *et al.*, 2004). One of these sigma factors that has been studied in detail is the alternative sigma factor SigF, which is similar to the general stress sigma factor, SigB, of *Bacillus subtilis* (DeMaio *et al.*, 1997). The gene encoding this sigma factor, *sigF*, is part of a gene cluster comprising *usfY* (unknown function), *usfX* (encoding a SigF-specific anti-sigma factor) and *sigF*, where *usfX* and *sigF* appear to be co-transcribed (Beaucher *et al.*, 2002; DeMaio *et al.*, 1997).

Deletion mutants of *M. tuberculosis* in *sigF* have been constructed and characterized. While none of the mutants was impaired in growth in broth culture or human macrophages *in vitro*, they showed reduced virulence in mouse and guinea pig infection models (Chen *et al.*, 2000; Geiman *et al.*, 2004; Karls *et al.*, 2006). Furthermore, it was shown that SigF was required for survival of *M. tuberculosis* in the lung, but not in the spleen of guinea pigs (Karls *et al.*, 2006). SigF did not appear to be required for survival of stress conditions such as heat and cold shock or long-term stationary-phase survival (Chen *et al.*, 2000). In fact, a recent study using both deletion as well as overexpression of *sigF* in *M. tuberculosis* suggested that SigF has a role in the regulation of the structure and function of the mycobacterial cell wall, rather than being a general stress-response sigma factor (Williams *et al.*, 2007).

The activity of SigF from *M. tuberculosis* is post-translationally regulated by the anti-sigma factor UsfX, which in turn is negatively regulated by two anti-anti-sigma factors, RsfA and RsfB, in response to different

Abbreviations: RACE, rapid amplification of cDNA ends; TSS, transcriptional start site.

physiological stress conditions (Beaucher *et al.*, 2002). In addition, several other proteins have been shown to interact with SigF or UsfX of *M. tuberculosis* and these are thought to exert some control on the activity of SigF (Parida *et al.*, 2005). There appears to be no consensus regarding the expression of *sigF* from *M. tuberculosis* in response to different stress conditions such as infection of macrophages (Graham & Clark-Curtiss, 1999; Mariani *et al.*, 2000), nutrient starvation (Betts *et al.*, 2002; DeMaio *et al.*, 1996; Michele *et al.*, 1999), stationary-phase growth (DeMaio *et al.*, 1996; Graham & Clark-Curtiss, 1999; Manganelli *et al.*, 1999), or exposure to oxidative stress, alcohol, antibiotics and temperature shock (DeMaio *et al.*, 1996; Manganelli *et al.*, 1999; Michele *et al.*, 1999).

When the gene encoding SigF was first identified in *M. tuberculosis*, the authors were able to show the presence of homologous genes in other slow-growing mycobacteria, but not in any of the fast-growers tested, including *Mycobacterium smegmatis* (DeMaio *et al.*, 1996, 1997). Inspection of the genome of *M. smegmatis* (<http://www.tigr.org>) revealed two open reading frames, MSMEG_1803 and MSMEG_1804, which have been annotated as *rsbW* (or *usfX*) and *sigF*, respectively, and recent literature has included SigF from *M. smegmatis* in discussions of mycobacterial sigma factors (Chowdhury *et al.*, 2007; Manganelli *et al.*, 2004; Waagmeester *et al.*, 2005). However, no experimental evidence is available regarding the role of SigF as an alternative sigma factor of *M. smegmatis*. The genome of *M. smegmatis* contains twice as many sigma factors as that of *M. tuberculosis* (26 versus 13), suggesting significant differences in the regulatory repertoire between these two species (Waagmeester *et al.*, 2005). However, while some sigma factors are unique to either *M. tuberculosis* (e.g. SigC, SigK and SigI) or *M. smegmatis* (e.g. multiple copies of SigH) and thus are likely to represent specific adaptations to the respective environments of these two species, several sigma factors, including SigF, are common to both (Waagmeester *et al.*, 2005).

In this paper, we report on the construction and characterization of a *sigF* deletion mutant of *M. smegmatis*. Furthermore, we used RT-PCR and transcriptional fusions to *lacZ* to study the expression of *sigF* in response to various stress conditions to determine whether *sigF* of *M. smegmatis* plays a similar role to the gene in *M. tuberculosis*.

METHODS

Bacterial strains and growth conditions. All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37 °C with agitation (200 r.p.m.). *Mycobacterium smegmatis* strain mc²155 (Snapper *et al.*, 1990) and derived strains were routinely grown at 37 °C, 200 r.p.m., in LB containing 0.05 % (w/v) Tween 80 (LBT), in Middlebrook 7H9 medium (Difco) supplemented with 10 % albumin-glucose-catalase enrichment (ADC; Becton Dickinson) and 0.05 % (w/v) Tween 80, or in modified Sauton's medium (ST). The composition of this medium per litre was as follows: 0.5 g MgSO₄ heptahydrate, 2 g citric acid monohydrate, 23 g K₂HPO₄ trihydrate,

0.3 g KCl, 1 g L-asparagine, 2 g glycerol, 0.5 g Tween 80, 320 µl 0.5 M FeCl₃ and 100 µl 1 M NH₄Cl. For carbon-limited growth, glycerol was reduced to 1 g l⁻¹; for nitrogen-limited growth, L-asparagine was reduced to 0.5 g l⁻¹. *M. smegmatis* transformants were grown at 28 °C for propagation of temperature-sensitive vectors and at 40 °C for allelic-exchange mutagenesis. Selective media contained kanamycin (50 µg ml⁻¹ for *E. coli*; 20 µg ml⁻¹ for *M. smegmatis*), gentamicin (20 µg ml⁻¹ for *E. coli*; 5 µg ml⁻¹ for *M. smegmatis*) or hygromycin B (200 µg ml⁻¹ for *E. coli*; 50 µg ml⁻¹ for *M. smegmatis*). Solid media contained 1.5 % agar.

Optical density was measured at 600 nm (OD₆₀₀) using culture samples diluted in saline to bring OD₆₀₀ to below 0.5 when measured in cuvettes of 1 cm light path length in a Jenway 6300 spectrophotometer.

DNA manipulation and cloning of constructs. All molecular biology techniques were carried out according to standard procedures (Sambrook *et al.*, 1989). Restriction or DNA-modifying enzymes and other molecular biology reagents were obtained from Roche Diagnostics or New England Biolabs. All primer sequences used are listed in Table 1.

Genomic DNA of *M. smegmatis* was isolated by a modification of the method of Gonzalez-y-Merchand *et al.* (1996) as described previously (Gebhard *et al.*, 2006). To create a construct for the deletion of *sigF*, the gentamicin resistance cassette (Gm^r), *aacC-1*, from pBSL141 (Alexeyev *et al.*, 1995) was excised using *EcoRI* and *SacI*. PCR products of approximately 820 bp flanking the *sigF* gene of *M. smegmatis* on either side were amplified using the primers sigFKO1 with sigFKO2, and sigFKO3 with sigFKO4, respectively. The left-flank PCR product was digested with *SpeI* and *EcoRI*, the right-flank product with *SacI* and *SpeI*. Both flanks and the gentamicin resistance cassette were ligated into the *SpeI* site of pBluescript II KS (Stratagene). The resulting assembly, left flank/Gm^r/right flank, was subcloned as a *SpeI* fragment into pPR23 (Pellic *et al.*, 1997), creating plasmid pSG45. The expected double-crossover event would result in a deletion-insertion at the *sigF* locus, eliminating 88 % of the *sigF* coding sequence in exchange for the gentamicin resistance marker (Fig. 1).

Allelic replacement of *sigF* was carried out essentially as described by Pellic *et al.* (1997). In brief, a culture of *M. smegmatis* carrying pSG45 was grown at 28 °C with agitation (200 r.p.m.) to an OD₆₀₀ of approximately 0.6–0.8, followed by plating onto low-salt LBT plates (2 g NaCl l⁻¹) containing gentamicin and 10 % sucrose at 40 °C, selecting for double-crossover events. To confirm the correct deletion of *sigF*, candidate colonies were screened by Southern hybridization analysis of *SacI*-digested genomic DNA. Replacement of *sigF* with the gentamicin resistance marker created strain SG128 (Δ *sigF::aacC-1*).

For complementation of the *sigF* mutation, a 1.8 kb PCR product encompassing both the *rsbW* and *sigF* genes plus 570 bp upstream DNA and 37 bp downstream DNA was amplified by PCR using primers csigFF and csigFR and cloned into the *HindIII* site of the integrative *E. coli*/mycobacteria shuttle vector pUHA267 (Lee *et al.*, 1991), creating plasmid pSG57. Transformation of strain SG128 with pSG57 resulted in strain SG158.

To create a transcriptional fusion of the *rsbWsigF* operon to *lacZ*, a 655 bp PCR product, encompassing 577 bp of DNA upstream of *rsbW* plus 78 bp of its coding region, was amplified using primers PsigFF and PsigFR. The product was cloned into the *ApaI* and *Asp718* sites of pJEM15 (Timm *et al.*, 1994), creating plasmid pAH55. Similarly, a transcriptional MSMEG_1802 fusion was created by amplification of a 426 bp PCR product encompassing 211 bp DNA upstream of MSMEG_1802 plus 215 bp of its coding region using primers P1802F and P1802R and cloning into the *ApaI* and *Asp718* sites of pJEM15, creating plasmid pAH59.

Table 1. Bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Description*	Source or reference
<i>E. coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80d <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>endA1</i> <i>nupG</i>	Hanahan <i>et al.</i> (1991)
<i>M. smegmatis</i>		
mc ² 155	Electrocompetent wild-type strain of <i>M. smegmatis</i>	Snapper <i>et al.</i> (1990)
SG128	mc ² 155 Δ <i>sigF::aacC-I</i> ; Gm ^r	This study
SG158	SG128 with pSG57 integrated in <i>attB</i> ; Gm ^r Hyg ^r	This study
Plasmids		
pBluescript II KS	Cloning vector; Ap ^r	Stratagene
pBSL141	<i>E. coli</i> plasmid containing an excisable <i>aacC-1</i> gene; Gm ^r Ap ^r	Alexeyev <i>et al.</i> (1995)
pPR23	<i>E. coli</i> -mycobacteria shuttle vector for allelic exchange mutagenesis in mycobacteria; Gm ^r Sac ^s ts	Pelacic <i>et al.</i> (1997)
pJEM15	<i>E. coli</i> -mycobacteria shuttle vector for the creation of transcriptional promoter fusions to <i>lacZ</i> ; Km ^r	Timm <i>et al.</i> (1994)
pUHA267	<i>E. coli</i> vector with mycobacteriophage L5 integrase and <i>attP</i> for integration into <i>attB</i> of mycobacteria; Hyg ^r	AgResearch, Wallaceville, NZ
pSG45	pPR23 harbouring Δ <i>sigF::aacC-I</i> ; Gm ^r Sac ^s ts	This study
pSG57	pUHA267 harbouring the <i>rsbWsigF</i> operon with its native promoter; Hyg ^r	This study
pAH55	pJEM15 harbouring a 570 bp <i>rsbW-lacZ</i> fusion; Km ^r	This study
pAH59	pJEM15 harbouring a 210 bp transcriptional <i>lacZ</i> fusion of MSMEG_1802; Km ^r	This study
Primers		
sigFKO1	AAATTTACTAGTGTACTCCGCGCTGAAGCACACGC	This study
sigFKO2	AAATTTGAATTCGTCGAGAACGTCTGCGTATTCCG	This study
sigFKO3	AAATTTGAGCTCAGATGCACGTCTCCCGTCTGCTG	This study
sigFKO4	AAATTTACTAGTTGCTACTCGTGGGCGGTGTGTG	This study
csigFF	AAATTTAAGCTTGGACAGCGGAATCGATACATGAAG	This study
csigFR	AAATTTAAGCTTAGGTTCCGACATGCCACCACTG	This study
rsbWRTF	CGTTCGAGGACCTCGACTTC	This study
sigFRTR	CCAGCAGACGGGAGACGTGC	This study
1802RTF	CCACTGGGAGGCAAAGAAGC	This study
PsigFF	AAATTTGGGCCCCGGACAGCGGAATCGATACATGAAG	This study
PsigFR	AAATTTGGTACCCACCACCGCCAGGTTCTCCAAC	This study
P1802F	AAATTTGGGCCCCGCTGCGGCGCCTTCTCATCTC	This study
P1802R	AAATTTGGTACCGCGACGCTTCTTGCCTCCCAGTG	This study
sigF-RACE1	AACGACACGAAGTCCGAG	This study
sigF-RACE2	CGACGATCTTCTCGCGCT	This study

*Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Hyg^r, hygromycin resistance; Ap^r, ampicillin resistance; Sac^s, sucrose sensitivity; ts, temperature sensitivity. Primer sequences are given in 5'–3' direction; restriction sites included in the primer sequences are underlined.

Stress conditions. Stress experiments were performed with mid-exponential-phase (OD₆₀₀ 0.7–1.0) cultures grown in LBT medium. For heat-shock (50 °C for 4 h) and cold-shock experiments (28 °C for 8 h), the cultures were placed at the indicated temperatures without further manipulation. For acid and alkaline stress, the cells were collected by centrifugation (16 000 g, 1 min) and resuspended to an OD₆₀₀ of 0.7 in citrate/phosphate buffer (100 mM, pH 4) or sodium borate buffer (13 mM, pH 9), respectively. For hyperosmotic and hypoosmotic stress, cells were collected as described above and resuspended to an OD₆₀₀ of 0.7 in 0.5 M NaCl or distilled water, respectively. For oxidative, alcohol and antibiotic stress, the respective compounds were added directly to the mid-exponential-phase culture. Hydrogen peroxide was tested at concentrations between 0.1 and 10 mM, rifampicin and isoniazid from 1 to 16 µg ml⁻¹ and D-cycloserine from 4 to 64 µg ml⁻¹. DMSO was included as a solvent

control, and had no effect on cell growth at all concentrations tested (up to 0.13 %). Cultures were incubated under stress conditions at 37 °C with agitation (200 r.p.m.) for 2 h or 4 h as stated. All stress experiments were carried out in triplicate. Survival was determined by viable cell counts from eight replicate spots of 5 µl per dilution. β -Galactosidase activities of strain AH56 (harbouring pAH55) were determined as described previously (Gebhard *et al.*, 2006) and were expressed as Miller units (Miller, 1972), calculated as the increase in A₄₂₀ per minute per 1 ml of cell suspension (normalized to an OD₆₀₀ of 1.0) used, and multiplied by a factor of 1000.

Statistical analyses were performed using paired or unpaired two-tailed *t*-tests for survival and β -galactosidase experiments, respectively. *P*-values are only reported where significant differences were found.

Fig. 1. The *sigF* gene locus and construction of a *sigF* deletion mutant of *M. smegmatis*. (a) Schematic of the *sigF* gene loci from *M. tuberculosis* (Mtb) and *M. smegmatis* (Msm). (b) Schematic of allelic replacement of *M. smegmatis sigF* with *aacC-1*. *SacI* restriction sites and fragment sizes as detected in (c) are indicated. The black bar shows the fragment used as a probe. (c) Southern hybridization analysis of replacement of *sigF* with *aacC-1*. *SacI*-digested genomic DNA from the wild-type (WT) and *sigF* deletion mutant SG128 ($\Delta sigF$) were probed with radiolabelled left-flank PCR product of the deletion construct.

Determination of *in vitro* killing by human neutrophils. Human neutrophils were isolated from heparinized peripheral blood from healthy adult donors using dextran sedimentation and centrifugation over a Ficoll-Hypaque gradient as described previously (Faldu *et al.*, 2002). Cells were washed with PBS buffer and remaining erythrocytes removed by hypotonic lysis with water. The isolated neutrophils were resuspended to a concentration of 4×10^7 cells ml^{-1} in RPMI 1640 medium supplemented with 10% autologous human serum or plasma. *M. smegmatis* strains were grown to an OD_{600} between 0.7 and 1.0 in LBT medium and diluted to 1.5×10^8 c.f.u. ml^{-1} in the same medium. Equal volumes of prepared mycobacterial cells and neutrophils (m.o.i. of 3.75) were diluted 10-fold into 1 ml prewarmed RPMI 1640 medium in 24-well plates and incubated at 37 °C and 5% CO_2 for 10 min, 20 min or 120 min. The neutrophils were lysed on ice for 20 min using 0.1% saponin, and survival of *M. smegmatis* was determined by viable cell count. As a control, the mycobacterial cells were incubated in RPMI 1640 medium without neutrophils.

Isolation of RNA and reverse transcriptase (RT)-PCR. RNA was extracted from 5 ml of broth culture grown to an OD_{600} of 0.5 in LBT medium. Cells were harvested by centrifugation (16 000 g, 1 min), washed in 1 ml GTC buffer [5 M guanidine thiocyanate, 0.5% (w/v) Sarkosyl, 0.5% (w/v) Tween 80, 100 mM 2-mercaptoethanol, 25 mM sodium citrate (pH 7)] and resuspended in 1 ml TRIzol reagent (Invitrogen). Cells were ruptured by two cycles of bead-beating with 0.5 ml zirconia beads (0.1 mm diameter) in a Mini-Beadbeater (Biospec) at 5000 r.p.m. for 1 min. RNA was isolated according to the manufacturer's instructions for the TRIzol reagent. The resulting RNA was resuspended in 50 μl DNase buffer [20 mM Tris/HCl (pH 7.5), 10 mM MgCl_2] and 20 U RNase-free DNaseI (Roche) were added, followed by incubation at 37 °C for 1 h. Final purification of RNA was performed using RNeasy kit spin columns (Qiagen) according to the manufacturer's instructions. RNA was quantified using a NanoDrop ND-1000 spectrophotometer. RT-PCR reactions were performed with the primers *rsbW*RTF and *sigF*RTF (Table 1) to determine whether *rsbW* and *sigF* were co-transcribed, and with primers 1802RTF and *sigF*RTF (Table 1) to detect a potential MSMEG_1802*rsbWsigF* transcript, using the Titan One Tube RT-PCR system (Roche) according to the manufacturer's instructions. The RT step was carried out at 55 °C for 30 min, followed by 94 °C for 2 min and 30 cycles of PCR consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 45 s for the *rsbWsigF* transcript or at 68 °C for 1 min for the MSMEG_1802*rsbWsigF* transcript. Final elongation was carried out

at 72 °C for 5 min. As a template, 150 ng (for the *rsbWsigF* transcript) or 220 ng (for the MSMEG_1802*rsbWsigF* transcript) of RNA was added per reaction. Control reactions with 150 ng RNA or 25 ng genomic DNA of *M. smegmatis* as template were performed using *Taq* DNA polymerase (Roche) according to the manufacturer's instructions.

Determination of transcriptional start sites (TSSs). The TSSs of *sigF* were mapped by 5'-RACE (rapid amplification of cDNA ends) using the components of the 3'/5'-RACE kit (Roche) according to the manufacturer's instruction. First-strand cDNA was synthesized from 5 μg total RNA of *M. smegmatis* with the *sigF*-specific primer *sigF*-RACE1. The resulting cDNA was purified and dA-tailed following the kit instructions. Purified dA-tailed cDNA was then used as a template for PCR using the Oligo dT-anchor primer and *sigF*-specific primer *sigF*-RACE2. The two resulting PCR products (550 bp and 1.1 kb) were gel purified and used as template for a second PCR using the PCR anchor primer and *sigF*-specific nested primer *sigF*KO2. These PCR products were cloned into pGEM-T Easy (Promega) according to the manufacturer's instructions. Three clones containing the correct-sized insert for both products were sequenced using primer *sigF*KO2 (500 bp product) or P1802R (1 kb product), respectively, and the last nucleotide before the poly-A tail to align with the genome sequence was chosen as the most likely TSS.

DNA and protein sequence analysis. Preliminary sequence data for *M. smegmatis* mc²155 were obtained from the Institute for Genomic Research website at <http://www.tigr.org>. Sequence data for *M. tuberculosis* were obtained from the Institut Pasteur website at <http://genolist.pasteur.fr/TubercuList>.

RESULTS AND DISCUSSION

Construction of a *sigF* deletion mutant of *M. smegmatis*

Sequence analysis of the region surrounding *sigF* of *M. smegmatis* showed that the gene is found in the same genetic locus as the homologous gene of *M. tuberculosis* (DeMaio *et al.*, 1997), downstream of its putative anti-sigma factor (*usfX* or *rsbW*) and adjacent to a gene encoding a probable bifunctional protein involved in

long-chain fatty acid synthesis (*bcc*) (Fig. 1a). However, *M. smegmatis* does not appear to contain a homologue to *usfY*, a gene with unknown function in *M. tuberculosis*, and neither MSMEG_1802 (annotated as ChaB protein of unknown function) nor MSMEG_1806 (conserved hypothetical protein) has sequence similarity to *UsfY*. BLASTP analysis (Altschul *et al.*, 1990) showed that SigF of *M. smegmatis* and *M. tuberculosis* are 78 % identical (data not shown).

To determine whether *sigF* of *M. smegmatis* encoded an alternative sigma factor with a similar function to SigF of *M. tuberculosis*, a *sigF* deletion mutant of *M. smegmatis* was created by allelic-exchange mutagenesis. A construct for the replacement of *sigF* with the gentamicin resistance marker (*aacC-1*) was cloned into pPR23 (Pelicic *et al.*, 1997) as described in Methods and transformed into *M. smegmatis* mc²155. Knockout mutants were selected as described previously (Pelicic *et al.*, 1997). The left flank of the deletion construct is located on a 2 kb *SacI* fragment (Fig. 1b). The coding region of *sigF* contains two *SacI* recognition sites. Replacement of *sigF* with *aacC-1* removes those *SacI* sites, while introducing a new site at the end of the gentamicin resistance cassette, resulting in a band shift from 2 kb in the wild-type to 2.9 kb in the deletion mutant (strain SG128) in Southern hybridization analysis of *SacI*-digested genomic DNA probed with a radiolabelled left-flank PCR product (Fig. 1b, c). While the fragments of both the wild-type and deletion mutant migrated slightly larger than expected, the difference between the two bands was consistent with the expected 900 bp shift (Fig. 1c).

In vitro phenotype of the *sigF* deletion strain

To study the general growth characteristics of the *sigF* deletion strain, SG128, in comparison to the wild-type, we monitored the growth of both strains in a number of different media. No differences in growth rate, length of lag phase or final optical density reached were seen between the wild-type and SG128 Δ *sigF* when grown in LBT, 7H9/ADC or ST minimal medium (limited for either nitrogen or carbon) (data not shown). A previously described *sigF* deletion mutant of *M. tuberculosis* H37Rv also showed similar growth to its parent strain *in vitro* (Karls *et al.*, 2006). In contrast, a *sigF* deletion mutant of *M. tuberculosis* CDC1551, while showing the same exponential growth rate, had a markedly decreased lag phase and grew to a threefold higher density as compared to the wild-type (Chen *et al.*, 2000). SigF therefore does not appear to play a role in exponentially growing cultures of *M. smegmatis*, consistent with the phenotype of the *M. tuberculosis* H37Rv *sigF* mutant (Karls *et al.*, 2006).

SigF has been described as a stress-response sigma factor of slow-growing mycobacteria (DeMaio *et al.*, 1996). We therefore wanted to investigate whether SigF was required for survival of the fast-growing *M. smegmatis* when exposed to various stress conditions. No significant differences between the *sigF* deletion strain and the

wild-type were detectable after 4 h exposure to alkaline pH (pH 9), hyperosmotic (0.5 M NaCl) or hypoosmotic (H₂O) shock or exposure to ethanol [5 % (v/v)] as determined by viable cell counts (Table 2). However, the *sigF* deletion mutant was significantly impaired in survival after 4 h of heat shock (50 °C) ($P < 0.05$) or exposure to acidic pH (pH 4) ($P < 0.01$), with a 10-fold and 20-fold reduction in viable cells, respectively, as compared to the wild-type (Table 2). Although no difference was seen between the wild-type and *sigF*-deletion mutant after 2 h exposure to hydrogen peroxide concentrations up to 5 mM, exposure to 7.5 mM hydrogen peroxide caused an approximately 100-fold decrease ($P < 0.005$) in viability of SG128 Δ *sigF* as compared to the wild-type (Table 2). Neither of the strains survived 2 h exposure to 10 mM hydrogen peroxide (data not shown). Complementation of the mutant, by supplying a single copy of the putative *rsbWsigF* operon to the cell (strain SG158), completely restored survival of both heat and acid stress as well as hydrogen peroxide stress (Table 2), thus confirming that the observed phenotype was solely due to the loss of SigF activity. For a previously described *sigF* deletion mutant of *M. tuberculosis*, no defect in survival of heat shock was observed, while survival at acidic pH or exposure to oxidative stress-generating agents had not been tested (Chen *et al.*, 2000). A similar phenotype of sensitivity to heat shock (52 °C) and oxidative stress (diamide or plumbagin) as seen here has been described for a *M. tuberculosis* mutant in a different alternative sigma factor,

Table 2. Survival of stress conditions by wild-type *M. smegmatis*, the *sigF* deletion strain (SG128) and the complemented strain (SG158)

Cells were exposed to stress conditions as detailed in Methods. Viable cell counts were performed immediately after exposure to the stress condition (t_0) and after 2 h (t_2) or 4 h exposure (t_4). Data are shown as the means $\pm$ SD of two or three independent experiments. ND, Not determined.

Stress condition		10 ⁻⁷ × Viable cell counts (c.f.u. ml ⁻¹)		
		Wild-type	SG128	SG158
Hypoosmotic	t_0	3.8 $\pm$ 2.4	2.7 $\pm$ 2.4	ND
	t_4	3.7 $\pm$ 2.5	1.8 $\pm$ 1.3	ND
Hyperosmotic	t_0	2.3 $\pm$ 1.3	1.3 $\pm$ 0.8	ND
	t_4	2.5 $\pm$ 1.1	1.6 $\pm$ 1.3	ND
pH 4	t_0	3.5 $\pm$ 0.9	2.7 $\pm$ 0.3	1.9 $\pm$ 0.5
	t_4	2.3 $\pm$ 1.1	0.14 $\pm$ 0.1	1.3 $\pm$ 0.5
pH 9	t_0	3.6 $\pm$ 0.9	2.3 $\pm$ 0.4	ND
	t_4	3.9 $\pm$ 0.7	4.1 $\pm$ 1.2	ND
5 % ethanol	t_0	3.3 $\pm$ 0.3	4.1 $\pm$ 1.1	ND
	t_4	8.6 $\pm$ 1.4	6.9 $\pm$ 0.6	ND
Heat (50 °C)	t_0	5.0 $\pm$ 0.9	4.1 $\pm$ 0.9	4.6 $\pm$ 0.5
	t_4	7.1 $\pm$ 0.5	0.51 $\pm$ 0.3	5.6 $\pm$ 3.4
H ₂ O ₂ (7.5 mM)	t_0	6.6 $\pm$ 0.9	6.4 $\pm$ 1.0	5.8 $\pm$ 0.9
	t_2	5.8 $\pm$ 1.6	0.055 $\pm$ 0.05	4.6 $\pm$ 0.8

SigH (Raman *et al.*, 2001). A *sigH* mutant of *M. smegmatis* also displayed increased sensitivity to oxidative stress by cumene hydroperoxide exposure, but no defect in survival of heat shock at 42 °C or 53 °C, or oxidative stress due to hydrogen peroxide exposure (Fernandes *et al.*, 1999). However, a *M. smegmatis* double mutant in SigH and another stress-response sigma factor, SigE, was impaired in survival of both heat shock and oxidative stress (cumene hydroperoxide) (Fernandes *et al.*, 1999). Raman *et al.* (2001) were able to identify a regulatory network between several stress-response sigma factors of *M. tuberculosis*, where SigH is responsible for stress-inducible expression of SigE, and both SigH and SigE are required for full induction of SigB. Considering the similar phenotype observed in the present study, it is conceivable that SigF of *M. smegmatis* either belongs to such a network of cross-regulation, or provides a separate, additional mechanism to respond to these stress conditions. This apparent redundancy of regulators for overlapping stress responses is further supported by the isolation of a *M. tuberculosis* mutant in yet another sigma factor, SigJ, which is also sensitive to hydrogen peroxide stress (Hu *et al.*, 2004). The requirement of SigF and SigH for resistance of *M. smegmatis* to oxidative stress is in accordance with the recent observation that the promoter of *dps*, a gene expressed under oxidative stress and starvation, can only be recognized by RNA polymerase containing one of these sigma factors (Chowdhury *et al.*, 2007).

Exposure to antibiotics is another condition that has been shown to induce expression of *sigF* in *M. tuberculosis* (Michele *et al.*, 1999), and a *sigF* deletion mutant had an increased susceptibility to rifamycin drugs (Chen *et al.*, 2000). We therefore determined the sensitivity of the *M. smegmatis* *sigF* mutant, SG128, to several antibiotics. No differences were seen in the MICs for rifampicin (4–8 µg ml⁻¹), isoniazid (4 µg ml⁻¹) or D-cycloserine (16–32 µg ml⁻¹) between strain SG128 Δ *sigF* and the wild-type. The MIC for rifampicin determined here for *M. smegmatis* was considerably higher than results obtained previously for *M. tuberculosis* (0.25 µg ml⁻¹) (Chen *et al.*, 2000). It is therefore possible that the relatively higher resistance of *M. smegmatis* to rifamycin antibiotics is responsible for the differences in phenotype of the respective *sigF* mutants. As discussed above, strain SG128 was more sensitive to killing by hydrogen peroxide, yet we observed no differences in its susceptibility to isoniazid as compared to the wild-type. Detoxification of hydrogen peroxide in mycobacteria involves two pathways, requiring the catalase-peroxidase KatG and the alkyl hydroperoxide reductase AhpC, respectively (Pagan-Ramos *et al.*, 1998). Both of these have been linked to altered susceptibility of the bacterium to isoniazid (Dhandayuthapani *et al.*, 1996; Heym *et al.*, 1993; Zhang *et al.*, 1992). It therefore appears that SigF of *M. smegmatis* regulates the response to hydrogen peroxide via a pathway independent of KatG or AhpC. Supporting this notion, expression of *ahpC* was not altered in the *sigF* deletion strain SG128 as compared to the wild-type (A. Hümpel & G. M. Cook, unpublished observations).

SigF is not required for survival of *M. smegmatis* in human neutrophils

As described above, the *sigF* deletion strain SG128 displayed a pronounced sensitivity to hydrogen peroxide exposure. While *M. smegmatis* is generally considered a non-pathogenic saprophytic soil bacterium, it has been shown to cause opportunistic skin infections and soft tissues lesions (Brown-Elliott & Wallace, 2002; Wallace *et al.*, 1988). We were therefore interested to determine whether strain SG128 Δ *sigF* displayed a reduced ability to survive in human neutrophils, which produce a wide range of oxidants, including hydrogen peroxide, when stimulated (Winterbourn *et al.*, 2006). No difference in survival was seen between either the wild-type and strain SG128 Δ *sigF* or the complemented strain SG158 when incubated with human neutrophils at an m.o.i. of 3.75 (data not shown), suggesting that SigF was not involved in survival of *M. smegmatis* in human neutrophils. This is in agreement with results obtained for a *M. tuberculosis* *sigF* mutant, which was not impaired in growth within human monocytes or in its susceptibility to lymphocyte-mediated growth inhibition (Chen *et al.*, 2000).

rsbW and *sigF* are co-transcribed in *M. smegmatis*

In *M. tuberculosis*, *usfX* and *sigF* are co-transcribed from a SigF-dependent promoter upstream of *usfX* (Beaucher *et al.*, 2002). To determine whether *rsbW* and *sigF* are also co-transcribed in *M. smegmatis*, we carried out RT-PCRs using primers that anneal to *rsbW* (*rsbW*RTF) and *sigF* (*sigF*RTTR), respectively (Fig. 2a, Table 1). Thus, a product can only be obtained if *rsbW* and *sigF* are co-transcribed. The expected size for a product was 1.02 kb. Using RNA isolated from an exponentially growing culture of *M. smegmatis* as template for RT-PCR, a product of the expected sizes was obtained (Fig. 2b, lane 2). Control PCRs performed without the reverse transcriptase step yielded no products (data not shown). These results show that *rsbW* and *sigF* of *M. smegmatis*, like those of *M. tuberculosis*, are co-transcribed.

As shown in Fig 1(a) and Fig. 2(a), an open reading frame (MSMEG_1802), annotated as *chaB* (unknown function) is present upstream of *rsbW* and transcribed in the same direction. To determine if this gene was also co-transcribed with *rsbW* and *sigF*, a further RT-PCR was carried out using primers annealing within MSMEG_1802 (1802RTF) and *sigF* (*sigF*RTTR), respectively, which would yield a 1.5 kb product from a potential MSMEG_1802*rsbW**sigF* transcript (Fig. 2a, Table 1). As shown in Fig. 2(c), a faint product of 1.5 kb was obtained, showing that there was indeed a transcript present containing all three cistrons.

To further characterize the *sigF* transcript, the TSS was determined by 5'-RACE analyses using a set of nested primers annealing within *sigF* (Table 1). Two products were obtained, ~500 bp and 1 kb in length, and the

Fig. 2. RT-PCR analysis of *rsbWsigF* transcription. (a) Schematic of RT-PCR reactions performed. Open triangles indicate the annealing position of the primers used within the *sigF* locus of *M. smegmatis*. Sizes of expected products for a *rsbWsigF* and *MSMEG_1802rsbWsigF* transcript are indicated. (b) RT-PCR for a potential *rsbWsigF* transcript. A 5 µl volume of the reaction was analysed by agarose gel electrophoresis and DNA was visualized by ethidium bromide staining. Lane 1, DNA marker (band sizes in kb are indicated on the left); lane 2, RT-PCR product from 150 ng RNA. (c) RT-PCR for a potential *MSMEG_1802rsbWsigF* transcript. A 10 µl volume of the reaction was analysed by agarose gel electrophoresis and DNA was visualized by ethidium bromide staining. Lane 1, DNA marker (band sizes in kb are indicated on the left); lane 2, RT-PCR product from 220 ng RNA.

corresponding TSSs were mapped to an adenyl residue 17 bp upstream of the *rsbW* start codon and a guanyl residue 41 bp upstream of the *MSMEG_1802* start codon, respectively (Fig. 3). It therefore appears that *sigF* of *M. smegmatis* is transcribed from two promoters, one upstream of *rsbW* (P_{rsbW}), and one upstream of *MSMEG_1802* (P_{MSMEG_1802}). Sequence analysis of P_{rsbW} revealed regions with similarity to the -10 and -35 elements of mycobacterial promoters (Agarwal & Tyagi, 2006), but no similarity to known SigF-dependent promoters from mycobacteria (Rodrigue *et al.*, 2007) (Fig. 3b). In contrast, P_{MSMEG_1802} contained -10 and -35 elements with strong similarity to the consensus sequence of SigF-dependent promoters of *M. tuberculosis* (GGWWT-N₁₆₋₁₇-GGGTAY) (Rodrigue *et al.*, 2007) (Fig. 3a).

Expression studies with transcriptional fusions to *lacZ*

In order to determine the activity of the two promoters identified above, transcriptional fusions of both regions to *lacZ* were created. The *rsbW-lacZ* construct (pAH55) contained 570 bp upstream of the start codon of *rsbW*, and the *MSMEG_1802-lacZ* construct (pAH59) contained 210 bp upstream of the start codon of *MSMEG_1802*. Cells of wild-type *M. smegmatis* harbouring pAH55 had β -galactosidase activities of around 55 Miller units (MU) when grown to mid-exponential phase in LBT medium (data not shown). Cells of wild-type *M. smegmatis* harbouring pAH59 displayed significantly lower levels of

Fig. 3. Analysis of promoter regions upstream of *sigF*. (a) P_{MSMEG_1802} region; (b) P_{rsbW} region. Transcriptional start sites were determined by 5'-RACE (traces are given as reverse sequence), and the most likely +1 nucleotides are indicated in bold. The start codons of *MSMEG_1802* and *rsbW*, as well as putative -10 and -35 elements of the promoters, are shown in bold; putative ribosome-binding sites (rbs) are underlined.

β -galactosidase activity ($P < 0.001$) of around 12 MU when grown to mid-exponential phase in LBT medium (Fig. 4).

As mentioned above, P_{MSMEG_1802} contained a sequence with similarity to SigF-dependent promoters of *M. tuberculosis*, while P_{rsbW} did not. To determine if any of these promoters were dependent on SigF in *M. smegmatis*, both transcriptional fusion constructs were introduced into the *sigF* deletion strain SG128. Strikingly, expression of *MSMEG_1802-lacZ* was reduced sixfold in strain SG128 as compared to the wild-type (Fig. 4a), with β -galactosidase activities of the mutant strain (2 MU) being similar to that of cells harbouring the empty vector pJEM15 (<2 MU, data not shown). These data strongly suggest SigF-dependence of P_{MSMEG_1802} . Complementation of the *sigF* deletion in strain SG158 restored expression to wild-type levels (Fig. 4a). In contrast, expression of *rsbW-lacZ* was unchanged in strain SG128 (data not shown), and P_{rsbW} therefore appears to be expressed independently of SigF.

To determine whether transcription from the P_{MSMEG_1802} or P_{rsbW} promoters was induced upon entry into stationary phase, *M. smegmatis* cells harbouring pAH55 and pAH59 were grown in LBT medium, and β -galactosidase activities were monitored throughout growth. For pAH55 (*rsbW-lacZ*) similar levels of activity (around 60 MU) were observed throughout all growth phases (data not shown). In contrast, expression from P_{MSMEG_1802} (pAH59) was induced approximately twofold (from ~12 MU to 23 MU) upon entry into stationary phase (Fig. 4b). These results are comparable to data of *M. tuberculosis sigF* in *Mycobacterium*

Fig. 4. Expression of MSMEG_1802-*lacZ*. (a) Effect of *sigF* deletion on expression. Cells of wild-type *M. smegmatis* (WT), the *sigF* deletion mutant SG128 ($\Delta sigF$) or the *sigF*-complemented strain SG158 ($sigF^+$) carrying the MSMEG_1802-*lacZ* construct pAH59 were grown to OD₆₀₀ 0.7–1.0 in LBT medium and β -galactosidase (β -Gal) activities, expressed in Miller units (MU), determined. Results are given as means $\pm$ SD of three technical replicates. Significant differences from the wild-type ($P < 0.05$) are indicated by two asterisks. (b) Expression throughout the growth cycle in LBT medium. Cells of *M. smegmatis* carrying pAH59 were grown in LBT medium, and OD₆₀₀ (■) and β -galactosidase activities, expressed as Miller units (grey bars) were monitored over time. β -Galactosidase activities are given as means $\pm$ SD of duplicate assays performed on each sample. Representative results from two independent experiments are shown.

bovis BCG, where 3.6-fold induction upon entry into stationary phase as compared to exponentially growing cells was observed (DeMaio *et al.*, 1996). However, the same study reported further induction (9.8-fold relative to exponential phase) in late stationary phase (DeMaio *et al.*, 1996), whereas β -galactosidase activities of AH60 in the present study remained at a similar level over 40 h after entry into stationary phase.

To determine whether the transcriptional fusion constructs of pAH55 and pAH59 were inducible in exponentially growing cultures by certain stress conditions, *M. smegmatis* cells harbouring these constructs were subjected to various stresses as described above. No change in *rsbW-lacZ* expression was seen after 2 h exposure to cold, mild heat shock (42 °C), ethanol, hydrogen peroxide, acidic or alkaline pH, hypoosmotic or hyperosmotic shock or exposure to rifampicin or isoniazid compared to unexposed control cells (data not shown). The only

condition tested which led to a change in *rsbW-lacZ* expression was exposure to 1 MIC of D-cycloserine (16 μ g ml⁻¹) causing a twofold induction ($P < 0.001$) from 53 MU to 100 MU. As a control, cells were also exposed to D-cycloserine in the presence of 200 μ g chloramphenicol ml⁻¹ to prevent any further synthesis of β -galactosidase. No induction compared to unexposed control cells was observed under these conditions (data not shown), confirming that the observed induction of *rsbW-lacZ* was indeed due to exposure to D-cycloserine. In *M. tuberculosis*, SigF appears to have a role in the regulation of genes involved in cell envelope biosynthesis (Geiman *et al.*, 2004; Williams *et al.*, 2007). It may therefore be that cell wall stress, e.g. due to exposure to D-cycloserine, causes an increase in expression of *sigF*, as observed here for *M. smegmatis*. The MSMEG_1802-*lacZ* construct did not show differential expression in response to any of the conditions tested here (data not shown). The general lack of induction of both constructs in response to most stress conditions tested is in accordance with previous results obtained after 2 h of stress exposure of *M. tuberculosis* (Manganelli *et al.*, 1999). However, taking into account the different growth rates of *M. smegmatis* and *M. tuberculosis*, the conditions chosen in the present study were thought to be more comparable to the 24 h exposure that DeMaio *et al.* (1996) used for their study of *M. bovis* BCG, where significant induction of *sigF* was observed in response to stress conditions such as oxidative stress, exposure to alcohol or cold shock. In the present and previous studies of the activity of SigF in mycobacteria, little if any regulation was observed at the transcriptional level (Beaucher *et al.*, 2002; Betts *et al.*, 2002; DeMaio *et al.*, 1996; Graham & Clark-Curtiss, 1999; Manganelli *et al.*, 1999; Mariani *et al.*, 2000), while more pronounced differences were seen at the translational and post-translational level (Beaucher *et al.*, 2002; Michele *et al.*, 1999). These findings support the argument that SigF activity may be regulated more strongly through the action of anti-sigma factors than through induction of *sigF* transcription (Beaucher *et al.*, 2002).

During the review process of this manuscript, a paper by Singh & Singh (2008) was published on the expression of *sigF* in *M. smegmatis*. The authors show that *usfXsigF* (synonymous with *rsbWsigF*) was expressed constitutively throughout the growth cycle and was not induced by the following stressors: rifampicin, streptomycin, NaCl, hydrogen peroxide and heat shock (45 °C), thus supporting the data presented here. However, *usfXsigF* was upregulated (<twofold) after 4–8 h exposure of exponentially growing cells to the following stressors: isoniazid, detergent (SDS), cold shock (15 °C), nutrient depletion (PBS), and ethambutanol (Singh & Singh, 2008). We did not test ethambutanol, PBS or SDS as inducers of *rsbWsigF* expression and the concentration of isoniazid used by Singh & Singh (2008) was 12-fold higher than that used in our study. Moreover, we routinely measured the transcriptional response of *rsbWsigF* after 2–4 h exposure to the test stressor and not the 4–8 h used in the Singh study.

In conclusion, our findings demonstrate that *sigF* of *M. smegmatis* encodes an alternative stress-response sigma factor, similar to its homologue from *M. tuberculosis*. Allelic-exchange mutagenesis showed that this sigma factor is required by *M. smegmatis* for the survival of heat shock, acid stress and oxidative stress. SigF-mediated resistance to oxidative stress most likely involves a KatG- and AhpC-independent pathway, because the *sigF* deletion strain displays unaltered sensitivity to isoniazid, and *ahpC* expression was unaltered in the *sigF* deletion mutant as compared to the wild-type (unpublished results). Despite its role in resistance to oxidative stress, SigF does not appear to be required for the survival of *M. smegmatis* in human neutrophils. RT-PCR and 5'-RACE analyses showed that the *sigF* locus of *M. smegmatis* had two promoters, one located upstream of *rsbW* and one upstream of MSMEG_1802. Transcription of MSMEG_1802-*lacZ* was SigF-dependent and induced twofold upon entry into stationary phase, but could not be induced in exponential phase after exposure to various stressors. Expression of *rsbW-lacZ* was independent of SigF and remained constant throughout the growth cycle and under various stress conditions unless cells were challenged with D-cycloserine.

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