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Role of the extracytoplasmic-function σ Factor σ^H in *Mycobacterium tuberculosis* global gene expression

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Summary

Like other bacterial species, *Mycobacterium tuberculosis* has multiple sigma (σ) factors encoded in its genome. In previously published work, we and others have shown that mutations in some of these transcriptional activators render *M. tuberculosis* sensitive to various environmental stresses and, in some cases, cause attenuated virulence phenotypes. In this paper, we characterize a *M. tuberculosis* mutant lacking the ECF σ factor σ^H. This mutant was more sensitive than the wild type to heat shock and to various oxidative stresses, but did not show decreased ability to grow inside macrophages. Using quantitative reverse transcription-PCR and microarray technology, we have started to define the σ^H regulon and its involvement in the global regulation of the response to heat shock and the thiol-specific oxidizing agent diamide. We identified 48 genes whose expression increased after exposure of *M. tuberculosis* to diamide; out of these, 39 were not induced in the *sigH* mutant, showing their direct or indirect dependence on σ^H. Some of these genes encode proteins whose predicted function is related to thiol metabolism, such as thioredoxin, thioredoxin reductase and enzymes involved in cysteine and molybdopterine biosynthesis. Other genes under σ^H control encode transcriptional regulators such as *sigB, sigE*, and *sigH* itself.

Introduction

Sigma (σ) factors are a class of proteins able to bind the RNA polymerase core enzyme and confer different promoter specificity on the resulting holoenzyme (Lonetto *et al*., 1992). Bacterial genomes usually encode one principal σ factor, responsible for the transcription of the housekeeping genes and a variable number of alternate σ factors that control responses to specific environmental stimuli.

ECF (extra-cytoplasmic function) σ factors represent a subfamily of alternate σ factors belonging to the σ^{70} class. Members of this subfamily are involved in regulating bacterial interactions with the extracellular environment, including adaptation to stress and, in some cases, bacterial virulence (Missiakas and Raina, 1998). The genome of *Mycobacterium tuberculosis* encodes 10 ECF σ factors (Gomez *et al*., 1997; Cole *et al*., 1998; Gomez and Smith, 2000). In previous work, we showed that the expression of *sigE* and *sigH*, both encoding ECF σ factors, was induced after heat shock, whereas the expression of *sigE* was induced also after surface stress (Manganelli *et al*., 1999). Recently, we disrupted the gene encoding σ^E (*sigE*) in *M. tuberculosis* H37Rv, and the mutant was more sensitive to a variety of oxidative stresses and heat shock as well as SDS-induced surface stress. Moreover, it was attenuated for growth in human macrophages and was more sensitive to the killing activity of activated murine macrophages. Using DNA microarray technology, we also studied the role of σ^E in the global gene expression profile of *M. tuberculosis* in response to SDS-induced surface stress. We found several genes whose induction after SDS treatment depended on the presence of a functional *sigE* gene. Among these genes were some encoding transmembrane heat shock proteins, enzymes involved in fatty acid degradation and transcriptional regulators such as *sigB*. Interestingly, *sigE* was shown to be required for the basal level of expression of *sigB* and for its induction after surface stress, but not after exposure to heat shock (Manganelli *et al*., 2001a).

In the *M. tuberculosis* genome, the σ factor encoded by *sigH* is the closest homologue of the *Streptomyces coelicolor* σ^R (Paget *et al*., 1998). In this related actinomycete σ^R-RNA polymerase is involved in oxidative stress response and is responsible for the transcription of both *sigR* and the *trx* operon, encoding thioredoxin and thioredoxin reductase in response to the thiol-specific oxidative agent diamide (Paget *et al*., 1998).

In this paper, we characterize a mutant of *M. tuberculosis* H37Rv obtained by disrupting the gene encoding σ^H. The *sigH* mutant strain was more sensitive than the wild

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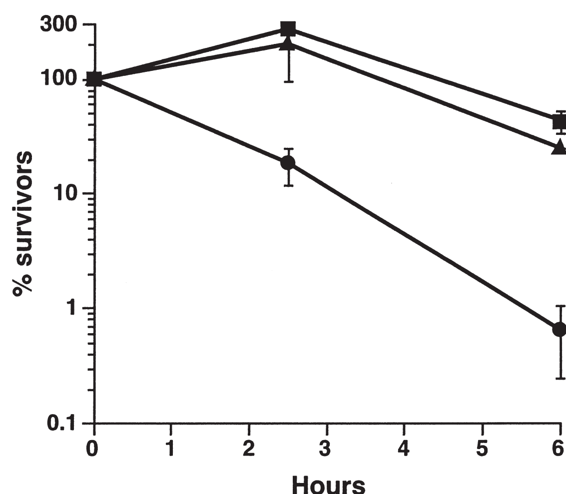

Fig. 1. Survival of *Mycobacterium tuberculosis* H37Rv and the *sigH* mutant ST49 after exposure to heat shock (45°C). The results are expressed as percentage colony-forming unit (cfu) with respect to T_0 (the beginning of the exposure to 45°C). The experiment, plated in triplicate, was repeated twice using independent mycobacterial cultures. The reported values represent the average and the standard deviation obtained for each point in one representative experiment. H37Rv (■), ST49 (●) and ST53, the complemented mutant (▲).

type to both heat shock and exposure to various oxidative agents, but was not attenuated in murine or human macrophages. Using quantitative reverse-transcription (RT-PCR), we were able to analyse the differences in the expression of selected genes in H37Rv and the *sigH* mutant during adaptation to oxidative stress caused by the thiol-specific oxidizing agent diamide and after heat shock. In addition, we used microarray technology to compare global expression profiles of the wild-type and *sigH* mutant strains after exposure to oxidative stress.

Results

Sensitivity of H37Rv and ST49 to environmental stresses

To determine the role of *sigH* in stress response, we constructed a null mutant (ST49) and a complemented strain (ST53) in *M. tuberculosis*, as described in *Experimental procedures*. We compared the sensitivity of H37Rv, ST49 and ST53 to heat shock and various oxidative stresses. ST49 was more sensitive than H37Rv to heat shock (Fig. 1), H_2O_2, cumene hydroperoxide and diamide (Table 1). On the other hand, its sensitivity to the superoxide generator plumbagine was the same as that of H37Rv. All phenotypes were restored in the complemented strain ST53 (Fig. 1 and Table 1).

Growth and survival of H37Rv and ST49 in macrophages

We previously demonstrated that the ECF σ factor σ^E is essential for the ability of *M. tuberculosis* to survive and multiply inside macrophages (Manganelli *et al*., 2001a). To see whether σ^H is also important for this ability of *M. tuberculosis*, we compared the growth of H37Rv and ST49 in both activated and not activated J774.1 murine macrophages, as well as in human THP-1-derived macrophages. No significant differences in growth could be detected between the two strains in these macrophage infections (data not shown).

Stress-mediated induction of sigB*,* sigH *and the* trx *operon*

The σ^H homologue in *Streptomyces coelicolor* (σ^R) responds to the thiol-specific oxidating agent diamide. In *M. tuberculosis*, sequences very similar to a σ^R promoter are present upstream of *sigB*, *sigH* and the *trx* operon (Paget *et al*., 1998; Gomez and Smith, 2000). These reports and the data shown in Fig. 1 and Table 1 suggest that σ^H responds not only to heat shock, but also to oxidative stress and thus could be involved in *sigB, sigH* and *trx* operon regulation in *M. tuberculosis*. To confirm this hypothesis, we compared the mRNA levels of *sigB*, *sigH* and *trxB2* (the first gene of the *trx* operon) in H37Rv and ST49 after heat shock and exposure to diamide, using quantitative RT-PCR. It is clear from the data obtained (Fig. 2) that both stresses caused an increase of *sigB, sigH* and *trxB2* mRNA levels and that this increase was σ^H-dependent. We were able to detect the *sigH* mRNA in the *sigH* mutant as the target sequence for the RT-PCR with molecular beacons was upstream of the site at which the kanamycin cassette was inserted into *sigH*. When the level of *sigE* mRNA was analysed in similar experiments, it increased both after diamide exposure and after heat

Table 1. Sensitivity of H37Rv, ST49 and ST53 to various oxidative stresses.

	H37Rv	ST49	ST53
H_2O_2 (5 μmol)[a]	1.7 ± 0.1[b]	3.6 ± 0.1	1.5 ± 0.1
Cumene hydroperoxide (350 nmol)[c]	3.6 ± 0.1	4.5 ± 0.1	3.6 ± 0.1
Plumbagine (100 nmol)[d]	2.2 ± 0.1	2.1 ± 0.1	2.3 ± 0.1
Diamide (20 μmol)	NA[e]	5.5 ± 0.3	NA

a. The values represent the amount of the inhibitory reagent added to the filter disc.
b. The reported values represent the average ± the standard deviation of the diameter of the inhibition zone in cm. The experiment, performed in triplicate, was repeated two times with independent bacterial cultures.
c. Cumene hydroperoxide comes as a DMSO solution. A negative control in which 10 ml of DMSO was added to the disc was performed: no inhibition of any of the three strains was detected.
d. Plumbagine was dissolved in 95% ethanol. A negative control in which 10 ml of 95% ethanol was added to the disc was performed: no inhibition of any of the three strains was detected.
e. No inhibition zone was detected.

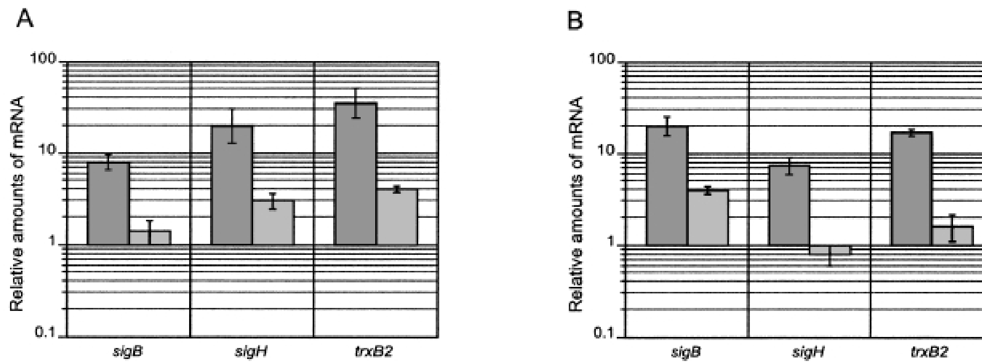

Fig. 2. Changes in *sigB*, *sigH* and *trxB2* mRNA levels after exposure to different stresses in *M. tuberculosis* H37Rv and the *sigH* mutant ST49. Levels of mRNAs after stresses were measured by molecular beacon reverse-transcription (RT)-PCR. Data are expressed as the ratio between the number of cDNA copies detected in samples obtained from the stressed culture, and the number of cDNA copies detected in samples obtained from unstressed bacteria. The values were normalized to the level of *sig*A mRNA, which was constant in both strains under these conditions (data not shown). Darker bars, H37Rv; lighter bars, ST49. A. Exposure to diamide (5 mM).
B. Heat shock (45°C).
The reported values represent the average and the error bars denote the range of the values obtained for each point in two separate experiments performed using two independent RNA preparations.

shock. However, this increase depended on the presence of a functional *sigH* gene only in the case of diamide exposure (data not shown).

Global expression profiles of H37Rv and ST49

To identify all genes in the σ^H regulon, we analysed the changes in the global expression profile of H37Rv and the *sigH* mutant ST49 after exposure to diamide.

A total of 48 genes was induced in the wild-type strain. Table 2 shows the expression levels of these genes before and after exposure to diamide in H37Rv depicted as fold-induction ratios. Similarly, the expression levels of these genes in ST49 before and after exposure to diamide are shown in the adjacent column. Genes for which the ratio of the H37Rv induction ratio compared with the ST49 induction ratio was 2.0 or greater were operationally defined to require σ^H for regulation, either directly or indirectly. Out of the 48 genes induced in the wild-type strain H37Rv by exposure to diamide, 39 were not induced in ST49, suggesting their dependence on σ^H. The remaining nine were induced in both strains. In agreement with the data previously obtained by quantitative RT-PCR, *sigB*, *sigE*, *sigH* and *trxB2* were shown to be induced by diamide and this induction was dependent on σ^H.

We also analysed changes in the *M. tuberculosis* global gene expression profile due to the absence of σ^H during standard physiological growth conditions (mid-exponential growth). In this case, we could not find any difference between H37Rv and ST49 (data not shown).

Search for a putative σ^H consensus sequence

Using the σ^R consensus sequence as a model, we (Gomez and Smith, 2000), Paget and colleagues (Paget *et al*., 1998), and Raman and colleagues (Raman *et al*., 2001) already predicted from sequence analysis that some *M. tuberculosis* genes (i.e. *Rv0991c*, *Rv2466c*, *sigB*, *sigH*, and *trxB2*), were under σ^H control. From our analysis, at least 15 out of the 27 putative transcriptional units found to be induced after diamide exposure in a σ^H-dependent manner have an ECF consensus sequence in their upstream region (Fig. 3). To provide evidence for this hypothesis, we initially performed RNA mapping experiments of the *sigB* promoter using primer extension analysis (Fig. 4). These assays show that the *sigB* transcriptional start site is directly downstream of the predicted σ^H promoter sequence. In other experiments, when *M. tuberculosis* was heat-shocked, the same transcriptional start site as determined by primer extension was used (data not shown). To extend this analysis, RNA mapping of *sigB* was done with 5′-RACE (rapid amplification of cDNA ends) (Frohman, 1994). These experiments showed that the same start site was used both in the wild type and the *sigH* mutant after diamide treatment (Fig. 5). This start site was identical to the one observed in exponentially growing cells as determined by primer extension (Fig. 4). 5′-RACE was also used to determine the start site of *Rv3463* in the wild-type strain before and after diamide treatment. This gene has also has an ECF consensus sequence in its upstream region (Fig. 3). We detected a transcript for this gene only after diamide treatment. Also in this case, the transcriptional start site was directly downstream of the predicted σ^H promoter sequence (Fig. 5).

The genes induced by diamide, but lacking a clear σ^H consensus sequence, are probably dependent on σ^H indirectly. We used 5′-RACE to map the promoter of two of

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Table 2. Global expression after diamide treatment.

Rv No.[a]	Gene	Ratio ↑ (H37Rv)	Ratio ↑ ST49	H37R versus ST49	Gene product[b,c]
0016c	*pbpA*	4.8 ± 1.0	3.6 ± 1.4	1.3	Penicillin-binding protein
0017c	*rodA*	2.5 ± 0.5	2.6 ± 1.4	1.0	FtsW/RodA/SpovE family protein
0141c*		9.3 ± 4.6	1.2 ± 0.1	**7.7**	HP
0142*		3.8 ± 0.8	1.1 ± 0.1	**3.4**	HP
0180c*		2.9 ± 0.6	1.1 ± 0.1	**2.6**	Probable membrane protein
0251c*	*hsp*	6.9 ± 4.3	1.9 ± 0.8	**3.6**	Possible heat-shock protein
0355c*	*PPE*	2.5 ± 0.4	1.1 ± 0.1	**2.3**	PPE-family protein
0384c*	*clpB*	5.6 ± 1.3	1.7 ± 0.3	**3.3**	Heat-shock protein
0740*		2.7 ± 0.7	1.1 ± 0.1	**2.4**	CHP
0991c*		3.8 ± 1.4	1.7 ± 0.4	**2.2**	HP
1130		2.9 ± 0.7	5.5 ± 1.5	0.5	CHP
1221*	*sigE*	5.7 ± 1.3	0.8 ± 0.1	**7.1**	ECF subfamily sigma subunit
	cysD	3.8 ± 1.1	3.4 ± 1.1	1.1	ATP:sulphurylase subunit 2
Rv1286	*cysN*	3.9 ± 0.6	3.1 ± 1.1	1.2	ATP:sulphurylase subunit 1
1334*		3.6 ± 1.4	0.9 ± 0.2	**4.0**	CHP
1335*		5.0 ± 1.0	1.3 ± 0.2	**3.8**	CHP
1336*	*cysM*	3.6 ± 0.9	0.9 ± 0.1	**4.0**	Cysteine synthase B
1337*		4.0 ± 0.4	1.0 ± 0.1	**4.0**	CHP
1338*	*murI*	3.3 ± 0.9	1.1 ± 0.1	**3.0**	Glutamate racemase
1471*	*trxB*	5.7 ± 1.1	1.0 ± 0.1	**5.7**	Thioredoxin reductase
1472*	*echA12*	4.2 ± 0.6	0.9 ± 0.1	**4.7**	Enoyl-CoA hydratase/isomerase protein
1528c*	*papA4*	8.6 ± 1.1	1.1 ± 0.1	**7.8**	PKS-associated protein
1645c*		2.6 ± 0.4	0.9 ± 0.1	**2.9**	CHP
1767		3.4 ± 0.9	5.0 ± 0.1	0.7	CHP
1874*		2.6 ± 0.4	1.2 ± 0.2	**2.2**	HP
Rv 1992c	*ctpG*	4.2 ± 1.1	4.2 ± 2.0	1.0	Probable cation transport ATPase
Rv 1993c		3.7 ± 0.5	4.6 ± 1.4	0.8	CHP
2397c*	*cysA*	3.2 ± 0.7	0.9 ± 0.1	**3.6**	Sulphate transport ATP-binding protein
2398c*	*cysW*	6.4 ± 2.1	1.1 ± 0.2	**5.8**	Sulphate transport system permease protein
2399c*	*cysT*	2.9 ± 0.9	0.9 ± 0.3	**3.2**	Sulphate transport system permease protein
2453c*		2.5 ± 0.4	1.1 ± 0.2	**2.3**	HP
2454c*		3.4 ± 0.5	1.2 ± 0.2	**2.8**	Oxidoreductase, beta subunit
2465c*	*rpi*	3.3 ± 0.7	1.2 ± 0.1	**2.7**	Phosphopentose isomerase
2466c*		10.4 ± 3.0	0.7 ± nd	**14.8**	CHP
2641		4.0 ± 1.2	7.2 ± 3.8	0.6	CHP
2698*		3.4 ± 0.9	1.1 ± 0.1	**3.1**	CHP
2699c*		3.5 ± 0.8	1.1 ± 0.1	**3.2**	CHP
2706c*		3.5 ± 0.9	0.9 ± 0.3	**3.9**	HP
2710*	*sigB*	2.6 ± 0.5	1.1 ± 0.3	**2.4**	RNA polymerase sigma subunit
3054c*		4.0 ± 1.0	1.3 ± 0.3	**3.1**	CHP
3206c*	*moeZ*	4.5 ± 1.0	1.0 ± 0.1	**4.5**	Probable molybdopterin biosynthesis protein
3222c*		4.9 ± 2.0	1.1 ± 0.2	**4.4**	CHP
3223c*	*sigH*	6.2 ± 1.7	1.0 ± 0.2	**6.2**	ECF subfamily sigma subunit
3463*		11.9 ± 4.3	1.3 ± 0.3	**9.1**	Probable neuraminidase
3464*	*rmlB*	2.6 ± 0.3	0.8 ± 0.1	**3.2**	dTDP-glucose 4,6-dehydratase
3465*	*rmlC*	3.9 ± 1.0	0.9 ± 0.1	**4.3**	dTDP-4-dehydrorhamnose 3,5-epimerase
3913*	*trxB2*	8.4 ± 2.0	1.3 ± 0.3	**6.5**	Thioredoxin reductase
3914*	*trxC*	5.7 ± 2.2	1.1 ± 0.2	**5.2**	Thioredoxin

A DNA microarray was used to measure the increase in gene-specific mRNA levels in *M. tuberculosis* cultures (H37Rv and ST49) after exposure to diamide 5 mM for 60 min. Ratios comparing RNA from a culture exposed to diamide to a log phase culture were calculated by averaging the data from six microarray experiments of three biological samples sets.

*Genes requiring σ^H for transcriptional regulation (directly or indirectly) were defined as having H37Rv/ST49 ratios equal to or greater than 2.0 (in bold).

a. H37Rv genes were included if their mRNA level was at least twofold greater in the diamide-treated H37Rv samples as compared with the untreated sample after subtraction of the standard deviation. All the ratios for these genes observed in the ST49 samples were included and used to calculate the H37Rv/ST49 ratios. Genes are listed in genomic order and are grouped if two or more genes in the same region of the genome fulfil the above criteria. Supplementary data for these and other DNA array experiments is available at http://schoolniklab.stanford.edu/projects/tb.html.

b. Genes are annotated as described by the Pasteur Institute on Tuberculist (http://genolist.pasteur.fr/Tuberculist).

c. (C)HP (conserved) hypothetical protein.

these genes (*papA4* and *rv2706c*) in the wild-type strain in the presence or absence of diamide induction. In both cases, we obtained a PCR product from the sample treated with diamide, which was absent in the uninduced sample. The resulting transcriptional start sites are also shown in Fig. 5. No similarity to any known promoter consensus sequence was observed in the region directly upstream of the transcriptional start sites for both genes.

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σR consensus
                 GGGAATnnnnnnnnnnnnnnnnn----cGTTg

hsp              gtGGAActtaagcgtggtcgactca---gGTTct    -94
Rv0355c          gcGGAActgtttgacagcgacgagacgctGTTgg   -229
clpB             gcGGAAcagactcaacattgacgg----cGTTga    -69
Rv0991c          ggGGAAtgacggaactcacctagc----gGTTct    -80
Rv1334           ccGGAAtaccgacctaccgtgaca----tGTTtc    -55
trxB             ctGGAAcaaagacttcatcctgagg---cGTTga    -36
Rv1645c          gcGGAccaaatcgtggacaacgcg----cGTTgt    -72
Rv2466c          cgGGAAcaggtgcggggcggccgt----gGTTgt    -77
Rv2699c          cgGGcAcgaatcgtttggtggacg----cGTTcg    -79
sigB             tgGGAActcaaagtccggctttgt----cGTTaa    -63
Rv3054c          tcGGAAtaaatcggaccatggtcc----gGTTgg    -72
sigH             ctGGAAataacccgctggcgaacac---gGTTga   -231
Rv3463           ccGGAAtagcaccggtgagccgagc---gGTTag    -37
moeZ             ggGGAAgcaaaggtggcaagctggg---cGTTga    -85
trxB2            cgGGAAcagcagtgcctacgctgg----cGTTcg    -65

                  c                           c   c
consensus          GGAAcnnnnnnnnnnnnnnnnn----  GTT
                  g                           g   g


Rv0142           gaGGctttgcaggaacatccaggcc---aGTTcg   -75
Rv1645c          gcGGAccaaatcgtggacaacgcg----cGTTgt   -71
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Fig. 3. Putative σ^H-specific promoters. The *consensus* promoter sequence recognized by RNA polymerase containing the *Streptomyces coelicolor* ECF sigma factor σ^R (shown in the first line) was used to search the upstream regions of genes induced in a σ^H-dependent manner after diamide treatment as determined by the DNA array analyses (Table 2). The consensus sequence calculated from the mycobacterial sequences illustrated in the figure is shown below the alignments. Putative –10 and –35 sequences are underlined; bases matching the mycobacterial consensus sequence are in bold. Bases of the consensus sequence conserved in more than 90% of the sequences are in capital letters. The two sequences shown below the consensus sequence have only partial similarity with the consensus sequence. Numbers on the right refer to the position of the first G of the conserved GGA sequence with respect to the start codon of the putative open reading frame (ORF).

Discussion

In several bacterial pathogens, transcriptional regulators have been shown to be essential for their virulence (Finlay and Falkow, 1997). The presence of genes encoding 13 different σ factors and 191 putative regulators in the *M. tuberculosis* genome (Cole *et al*., 1998) suggests an important role for some of these transcriptional regulators in this bacterium's ability to survive and grow in the infected host. Recently, strains of *M. tuberculosis* lacking the genes encoding the σ factors σ^E (Manganelli *et al*., 2001a) and σ^F (Chen *et al*., 2000) have been shown to be attenuated. To understand the complex regulatory networks of *M. tuberculosis* and to study their roles in virulence, we recently started the characterization of the σ^E regulon using DNA array technology (Manganelli *et al*., 2001a).

In this paper, we characterize a mutant of *M. tuberculosis* H37Rv in which the gene encoding the ECF s factor σ^H was disrupted. σ^H is a very close homologue of the *S. coelicolor* σ factor σ^R (Paget *et al*., 1998). The activity of this σ factor is regulated in *S. coelicolor* at the post-translational level by an anti-σ factor (RsrA) that ordinarily binds σ^R and keeps it in an inactive form. The thiol-specific oxidizing agent diamide catalyses the formation of disulphide bridges in RsrA, causing a conformational change in this protein so it can no longer bind to σ^R. The now active σ^R, after association with core RNA polymerase transcribes its own structural gene *sigR* as well as the operon encoding thioredoxin and the thioredoxin reductase (Kang *et al*., 1999; Paget *et al*., 2001a). The consensus sequence for the σ^R-RNA polymerase has

A

G G G G T A ▶C T G T C A

A C G T

B

-35 -10

TCACTGCTGGGT**GGGAAC**TCAAAGTCCGGCTTTGT**CGTTAA**ACCCCAT**G**ACAGTGCAAGCCG

Fig. 4. Identification of σ^H transcriptional start sites. The 5′-terminus of the *sigB* transcript was determined by primer extension using an oligonucleotide that was complementary to the postulated *sigB* transcript and RNA prepared from *M. tuberculosis* H37Rv grown in 7H9 + ADN in the mid-exponential phase of growth.
A. The lanes marked ACGT are the sequencing ladder that was made with the same primer used for the primer extension and plasmid pSM110 that contains the intact *sigB* gene and several kb upstream. The bottom strand sequence is shown to the left of the figure and the arrow indicates the position of the transcriptional start-point (TSP). The rightmost lane adjacent to the sequencing ladder shows the migration of the primer extension product.
B. The top strand sequence and the –10 and –35 regims are highlighted in bold. The arrow indicates the TSP.

sigB
TCACTGCTGGGT**GGGAAC**TCAAAGTCCGGCTTTGT**CGTTA**AACCCCAT**G**ACAGTGC –28
rv3463
CCAGCGCTCAC**CGGAAT**AGCACCGGTGAGCCGAGC**GGTTA**GAGCAACC**A**TGACCAA +1
papA4
TCGAAGACGCGACCACGGCAGCACACTACCTCTAACGCTTCGTCAACG**C**TGACGGT –19
Rv2076c
TCGCTCCCGCTGACCTCCAGCACGGCGGACATCTCGGTCAGTTTCGCC**T**CGTGCTC –2

Fig. 5. Identification of σ^H-dependent TSPs. The TSPs of genes that required a functional *sigH* for their expression after diamide treatment was determined by 5′ RACE determinations as described in the *Experimental procedures* section. The sequence of the top strand of the *sigB*, *rv3463, papA4* and *rv2706c* upstream regions is shown, and the TSP is indicated by the nucleotide in bold type. Numbers on the right refer to the position of the transcriptional start site with respect to the start codon of the putative ORF. For the *sigB* and *Rv3463* genes, conserved sequences that resemble the extra-cytoplasmic function (ECF) consensus in the –10 and –35 regions of these promoters are indicated by bold letters. *papA4* and *Rv2706c* did not have an ECF consensus sequence.

been described (Paget *et al*., 1998) and is found upstream of several mycobacterial genes such as *sigH*, *sigB* and *trxB2* (Gomez and Smith, 2000; Paget *et al*., 2001b; Raman *et al*., 2001). Moreover, downstream of *sigH* is a gene encoding a protein that shows high similarity to RsrA. These data suggest that σ^H is the σ^R homologue in *M. tuberculosis*. The finding that *sigH*, as well as *sigB* and *trxB2* were induced in a σ^H-dependent manner after both heat shock and diamide exposure (Fig. 2), confirmed this hypothesis. Interestingly, no information is available concerning a possible role of σ^R in the *S. coelicolor* heat-shock response.

The *sigH* mutant was shown to be more sensitive than the wild type to heat shock (Fig. 1) and various oxidative agents such as H_2O_2, cumarine hydroperoxide and diamide, but not to the superoxide generator plumbagine (Table 1). This is in contrast with the data recently published by Raman and colleagues (Raman *et al*., 2001), which show a sensitivity to plumbagine for a similar *sigH* mutant. All the phenotypes were completely complemented when a wild-type copy of *sigH* was integrated at the mycobacterial phage L5 *attB* site. Surprisingly, despite its sensitivity to oxidative stress, the *sigH* mutant's ability to grow inside non-activated macrophages and to survive inside activated macrophages was the same as that shown by the wild-type strain H37Rv. Preliminary results also indicate that the *sigH* mutant shows no impairment in growth during mouse infections (R. Manganelli, L. Fattorini and I. Smith, unpublished results). It is possible that σ^H does play a role in virulence but that its functions can be assumed by redundant mechanisms, such as other ECF σ factors. This possibility can be tested by studying the virulence of *M. tuberculosis* strains that carry mutations in multiple ECF σ factor genes.

A DNA microarray containing 97% of the predicted open reading frames (ORFs) of the *M. tuberculosis* genome was used to compare the basal gene expression in the wild-type and the *sigH* mutant strain growing in exponential phase. In this condition, no significant difference was found between the two strains, suggesting that σ^H does not play any role in *M. tuberculosis* physiology during standard growth conditions. In contrast, we found variations in the basal level of expression of 41 genes when similar experiments were performed to compare the σ^E mutant to the wild-type strain (Manganelli *et al*., 2001a).

We identified 48 genes induced in H37Rv after diamide exposure. Most of these genes (39) were not induced in the *sigH* mutant, indicating that their induction was directly or indirectly dependent on σ^H. Out of the 39 genes, 21 are annotated to encode proteins of known function. It is possible to place these proteins into different functional categories: (i) *hsp* and *clpB* encode two heat-shock proteins; (ii) *sigB*, *sigE*, *sigH* and *rv0142* encode three σ factors and a putative transcriptional regulator. Interestingly, *hsp* and *sigB* were also induced in a *sigE*-dependent manner after surface stress (Manganelli *et al*., 2001a); (iii) the *trxB2C* operon encodes a thioredoxin reductase and a thioredoxin and *trxB* encodes a second thioredoxin. It is interesting to note that upstream of *trxB* is a third gene encoding a thioredoxin (*trxA*), but this is not induced by diamide; (iv) *cysA*, *cysW*, *cysT* and *cysM* encode proteins involved in cysteine biosynthesis; (v) *rpi* encodes a ribose phosphate isomerase and *rmlB* and *rmlC* encode enzymes involved in glucose metabolism.

The redox status of the sulphydryl groups of the cysteine residues can affect structure and function of many proteins. The intracellular milieu is usually a reducing environment, but reactive oxygen species or other oxidative compounds can alter this redox balance (Aslund and Beckwith, 1999). The thioredoxin/thioredoxin reductase and the glutathione/glutathione reductase systems are usually among the main systems implicated in the regulation of the redox homeostasis (Grant, 2001). Streptomycetes and mycobacteria produce a low molecular weight thiol: mycothiol (1-D-*myo*-inosityl-2-(*N*-acetyl-L-cysteinyl)amino-2-deoxy-α-D glucopyranoside) instead of glutathione (Newton *et al*., 1996). The biosynthetic pathway for this complex molecule has not yet been completely defined (Newton *et al*., 2000). The induction of enzymes involved in cysteine biosynthesis, and in metabolism of ribose and glucose, could indicate an increased need for the precursor of mycothiol biosynthesis. In support of this hypothesis, recently Paget and colleagues (Paget *et al*., 2001b) showed that a mutant of *S. coelicolor* lacking the σ^H homologue σ^R produces four times less mycothiol than the wild type. Moreover, the σ^H-dependent induction of a gene whose product contains a probable glutaredoxin active site (Rv2466c) suggests that this protein could be part of the oxidoreductive chain involved in protein disulphide reduction; (vi) *rv1335*, *rv2453c* and

moeZ encode enzymes that are probably involved in the biosynthesis of molybdopterin, a dithiol-containing cofactor required by a number of molybdo-enzymes.

The *S. coelicolor* σ^R consensus sequence was used to search the upstream sequence of the 39 genes that require σ^H for induction after diamide stress. In 15 of these genes, we were able to identify a sequence similar to that recognized by the *S. coelicolor* σ^R (Fig. 3) and for two of these genes, *sigB*, and *Rv3463*, the assignment of this sequence as a promoter was validated by primer extension analysis and by 5′-RACE (Figs 4 and 5).

We previously showed that most of the basal level of *sigB* transcription and its induction after surface stress (but not after heat shock) depended on the presence of a functional *sigE* (Manganelli *et al*., 2001a). However, in this communication we show that *sigB* induction after heat shock and after diamide exposure are *sigH*-dependent. From these data, we can infer that *sigB* is transcribed either from the σ^E-RNA polymerase or from the σ^H-RNA polymerase depending on the physiological conditions: the σ^E-RNA polymerase would be responsible for *sigB* transcription during standard (unstressed) growth conditions and after surface stress (Manganelli *et al*., 2001a), whereas the σ^H-RNA polymerase would be responsible for its transcription after heat shock. Either polymerase might be directly responsible for *sigB* transcription following exposure to diamide as both *sigE* and *sigH* are induced by this stress, in a σ^H-dependent mechanism. In a recent publication, Raman and colleagues (Raman *et al*., 2001) reached similar conclusions.

The fact that *sigE* does not have a σ^H recognition motif in its promoter, unlike *sigH* indicates that its dependence by σ^H is indirect. Raman and colleagues (Raman *et al*., 2001), recently reported that *sigE* induction after diamide stress was due to transcription from a transcriptional start site downstream of a σ^H-specific consensus sequence. The fact that this transcriptional start site is 61 bp internal to the predicted *sigE* ORF suggests either an artefact or a truncated protein being translated after diamide stress in their experiments.

It is worth noting that the same promoter is used for *sigB* transcription during standard growth conditions and after diamide treatment (Figs 4 and 5), suggesting that both σ^E- and σ^H-RNA polymerase recognize the same promoter. The fact that the *sigB* promoter resembles both the *sigE* (Manganelli *et al*., 2001a) and the *sigH* consensus promoter sequences supports this hypothesis. We currently favour the hypothesis that most, if not all, of the diamide induction of genes that have the σ^H/σ^E-like promoter sequence are actually transcribed by σ^H containing RNA polymerase. The main reason for this is that only two genes that require σ^E for their expression after SDS stress, Rv0251c (*hsp*) and *sigB* out of 38 (Manganelli *et al*., 2001a) are induced after diamide stress (Table 2).

```
papA4    CGC---GACCACGG-------CAGCAC
rv2706c  CGCT--GACCTC---------CAGCAC
rv0141c  CGCGGTGACCGGCCCGGTGGGCAGCAA
cysT     GGC---GACCgg---------CAGCAT
```

Fig. 6. Potential regulatory sequences. Sequences found upstream of the *papA4*, *rv2706c*, *rv0141c* and *cystT* genes were aligned and conserved sequences are highlighted in bold letters. These genes did not have an ECF consensus sequence.

This suggests that the putative anti-σ factor that controls σ^E activity is still active after diamide stress. Additional DNA array studies and transcription experiments with purified RNA polymerases will be necessary to demonstrate this hypothesis and these studies are currently in progress.

Some of the genes induced by diamide in a σ^H-dependent manner lacked a putative σ^H promoter. We characterized the transcriptional start site of two of these genes (*papA4* and *rv2706c*). An alignment of the two regions upstream of the transcriptional start site revealed the presence of a conserved sequence starting at position –25 (*papA4*) and –26 (*Rv2706c*) (Fig. 6). Similar conserved sequences were also found upstream of two other genes belonging to the same class, *rv0141c* and *cysT* (Fig. 6), suggesting that the transcription of these genes could be under the control of a novel regulator whose expression is under σ^H control.

We believe that regulation of gene expression is of great importance for the virulence of *M. tuberculosis*. In previous work, we started to characterize the complex regulatory network involved in stress response and virulence due to the ECF sigma factor σ^E (Manganelli *et al*., 2001a). In the present work, we continue this work of characterizing *M. tuberculosis* global gene regulation, studying a mutant lacking the ECF σ factor σ^H. We have shown that it is involved in the response to oxidative stress and heat shock, and have begun an identification of the genes that are in its regulon.

Experimental procedures

Bacterial strains, media and growth conditions

All experiments other than plasmid constructions were performed with *M. tuberculosis* H37Rv and its derivatives obtained during this study. Bacteria were grown either in Middlebrook 7H9 (liquid medium), or in 7H10 (solid medium) (Difco), both supplemented with ADN (albumin, dextrose and NaCl) (Manganelli *et al*., 2001b), 0.2% glycerol and 0.05% Tween 80. Liquid cultures were grown in roller bottles at 37°C. Plates were incubated at 37°C in sealed plastic bags.

Escherichia coli strain JM109 was grown in Luria–Bertani broth (LB, Difco) at 37°C with agitation. Antibiotics, when required, were added at the following concentrations: kanamycin, 20 μg ml^{-1} in *M. tuberculosis* or 50 μg ml^{-1} in *E. coli*; hygromycin, 150 μg ml^{-1} in *M. tuberculosis* or 50 μg ml^{-1}

in *E. coli*; ampicillin ($100\ \mu g\ ml^{-1}$); streptomycin ($20\ \mu g\ ml^{-1}$). Sucrose selection was performed on 7H10 plates with 8% sucrose.

DNA manipulations

All recombinant DNA techniques were performed following standard procedures, using *E. coli* JM109 as a host. DNA restriction and modifying enzymes were obtained from Promega and used according to the manufacturer's suggestions. PR50 (5′-ATCGGCGGGGACAGCGTCAG-3′) and PR51 (5′-TCACTTCCGCGCAACCCATG-3′) were used to amplify the 2022 bp fragment containing *sigH* used for *sigH* disruption; PR50 and PR71 (5′-GCTGGCAAGACGGCATCG CTTAC-3′) were used to amplify the 1496 bp fragment used to construct the complemented strain.

sigH *disruption and complementation*

We cloned a 2022 bp PCR fragment containing the *sigH* gene in a suicide vector (pSM270, unpublished, sequence and map available on request), which contains both *sacB* (conferring sucrose sensitivity) and a cassette conferring streptomycin resistance. The *sigH* gene was then disrupted by inserting a cassette conferring kanamycin resistance into the unique *Kpn*I site internal to the *sigH* gene. This construct was electroporated into *M. tuberculosis* H37Rv, with selection for kanamycin resistance, followed by selection for sucrose resistance, which would result from the loss of the plasmid backbone containing the *sacB* gene (Pelicic *et al.*, 1996). Kanamycin-resistant, sucrose-resistant strains were then tested for streptomycin sensitivity to confirm the loss of the plasmid and analysed by Southern blotting following standard techniques (Sambrook *et al.*, 1989).

To complement the *sigH* mutant, we cloned a PCR fragment containing the 1496 kb *sigH* gene fragment in the integrative vector pYUB413 (V. Balasubramanian and W. Jacobs, unpublished). This vector contains the *attP* site and the gene encoding the integrase of the mycobacteriophage L5 and it is able to integrate at the unique L5 *attB* site in the genome of *M. tuberculosis* (Hatfull and Sarkis, 1993). pYUB413 contains a cassette conferring resistance to hygromycin. The pYUB413 derivative carrying the *sigH* gene was electroporated into the *sigH* mutant ST49 with selection for hygromycin resistance, giving strain ST53.

Electroporation of M. tuberculosis

Electroporation of *M. tuberculosis* was performed as described previously (Manganelli *et al.*, 2001a). Briefly, a bacterial culture in mid-exponential phase was washed twice in 10% glycerol and resuspended in 1/100 of the initial volume. A $70\ \mu l$ aliquot of the cell suspension was mixed with $10\ \mu g$ of transforming DNA and loaded into a Disposable Cuvette Plus (0.2 cm electrode gap) (BTX). The sample was subjected to a single pulse using the Electroporator 2510 (Eppendorf) (capacitance, 10 mF; voltage $12.5\ kV\ cm^{-1}$; resistance 600 W). After the pulse, the cells were diluted in 1 ml of 7H9, incubated for 24 h at 37°C, and then plated on selective solid medium.

Heat shock induction

Bacteria were exposed to heat shock as described previously (Manganelli *et al.*, 1999). Briefly, an exponentially growing culture was divided into two 10 ml aliquots: one aliquot was incubated in a waterbath at 45°C; the other aliquot was incubated at 37°C, and both incubations were for 60 min. The bacteria were then chilled on ice, centrifuged at 3000 *g* for 3 min at 2°C, resuspended in 1 ml of cold LETS buffer (100 mM LiCl; 10 mM EDTA; 10 mM Tris, pH 7.8; 1% SDS), and the cell pellets were frozen on dry ice and stored at –70°C until they were used to prepare RNA.

Diamide induction

A bacterial culture in the mid-exponential phase of growth was divided into two 10 ml aliquots. Diamide was added to one of the aliquots to a final concentration of 5 mM. After 60 min of incubation at 37°C, bacteria were chilled on ice, centrifuged at 3000 *g* for 3 min at 2°C, resuspended in 1 ml of cold LETS buffer, and the cell pellets were frozen on dry ice and stored at –70°C until they were used to prepare RNA.

RT-PCR with molecular beacons

RNA extraction was performed as described previously (Manganelli *et al.*, 2001b). The primers and beacons for *sigA* and *sigB* were described in a previous work (Manganelli *et al.*, 1999). The primers specific for *sigH*, and *trxB2* are the following: *sigH* 5′-GCAGCCTGGGCCGTCTGA-3′ (upper) (5′-GGCCGGATTGCGCGTCAT-3′ (lower); *trxB2* 5′-TACACT GCGGCGCTCTAC-3′ (upper); 5′-CACGTCGGTGGTGGT CAT-3′ (lower). The sequences of the molecular beacons for *sigH* and *trxB2* were, respectively: Fluorescein-5′-GGAC GCGCGATTCCCCTGTTGGACCA GCGTCC-3′-DABCYL (*sigH*); Fluorescein-5′-GGACCC AGGGCACGTCTTTCGGC GG GGGTCC-3′-DABCYL *(trxB2*). The complementary arms of the generic stem are underlined. Reverse transcription was performed as described previously (Manganelli *et al.*, 2001b) using Avian Myeloblastoma Virus Retro-Transcriptase (AMV) (USB). PCR with molecular beacons was performed as described previously (Manganelli *et al.*, 2001b) using an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler (Perkin-Elmer) and AmpliTaq Gold polymerase (Perkin-Elmer).

Quantitative analysis of the data was performed as described previously (Manganelli *et al.*, 2001b). Results were normalized to the amount of *sigA* mRNA which was shown to be constant in all the samples tested (data not shown).

Primer extension analysis and RACE

Primer extension analysis to map the *sigB* promoter was performed as described previously (Dussurget *et al.*, 1999; Rodriguez *et al.*, 1999), using RNA extracted from *M. tuberculosis* H37Rv grown in liquid media by mechanical disruption with glass beads and phenol extraction.

5′ RACE experiments were performed using the 5′/3′ RACE Kit (Roche Molecular Biochemicals) following the manufacturer's suggestions. Briefly, exponentially growing

M. tuberculosis H37Rv and the sigH mutant ST49 cultures growing in 7H9-ADN medium were treated with 5 mM diamide as described earlier in this section and RNA was prepared as previously described (Manganelli *et al*., 1999). cDNAs were made with AMV reverse transcriptase using 1 µg of RNA and gene specific reverse primers that were generally 100–150 bp downstream from the translation initiation codon of the genes. cDNAs were poly dA tailed at their 3′-ends with terminal transferase and they were PCR amplified after a 'hot start' with *Taq* polymerase, using a poly dT primer provided in the kit that was complementary to the 3′-tail and a second gene-specific primer that was nested upstream of the original cDNA primer. The resulting amplicons were PCR-amplified a second time with the same poly dT primer and a second nested primer that was internal to the first. The PCR amplification products were fractionated by agarose gel electrophoresis and they were cloned using the TOPO TA Cloning Kit (Invitrogen). Plasmid clones resulting from *E. coli* transformation were then sequenced using the M13 forward and reverse primers that are complementary to DNA sequences adjacent to the TA cloning site of the kit's cloning vector pCR2.1-TOPO. Several PCR clones for each RACE determination were sequenced in this manner, and they generally gave identical transcriptional start sites. Sequences for all of the primers used in the RACE assays are available on request.

Killing curves after heat shock

Mycobacterial strains were grown to early exponential phase and plated on 7H10 solid media to determine viable cell number (T_0). The culture was incubated in a waterbath at 45°C. At different times, 50 µl samples were diluted in 7H9 and plated to determine the number of colony-forming units (cfu). Results were expressed as percentage survivors with respect to T_0.

Determination of growth inhibition by zone diffusion assay

Mycobacterial strains were grown to early exponential phase. Aliquots of 100 µl containing 3×10^6 cfu were spread on 7H10 plates. Paper discs (6.5 mm in diameter) (Schleicher Schuell) containing 10 µl of the inhibitory reagent were placed on top of the agar. Diamide was dissolved in H_2O, plumbagine in 95% ethanol. Cumene hydroperoxide comes as a solution in DMSO and was diluted in H_2O. Negative controls with DMSO and 95% ethanol were performed. The diameters of the zones of inhibition were measured after 15 d of incubation at 37°C.

Microarray analysis

RNA extraction was performed as described previously (Manganelli *et al*., 2001b). Steps in *M. tuberculosis* DNA microarray gene expression analysis were performed as described by Schoolnik and colleagues (Schoolnik *et al*., 2001) using a DNA microarray representing 97% of the *M. tuberculosis* ORFs. Briefly, cDNA, made from two RNA samples labelled with either Cy3- or Cy5-fluorochromes (Amersham Pharmacia Biotech), were combined and hybridized to the microarray. The microarray was washed and then scanned using the ScanArray 5000 (GSI Lumonics). The intensities of the two dyes at each spot were quantified using SCANALYZE written by Michael Eisen at Stanford University and available at http://rana.stanford.edu/software/. The results of the DNA array analyses reported in this paper are available at the following site:http://schoolniklab.stanford.edu/projects/tb.html.

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