

Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*

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Summary

The ability of *Mycobacterium tuberculosis* to adapt to different environments in the infected host is essential for its pathogenicity. Consequently, this organism must be able to modulate gene expression to respond to the changing conditions it encounters during infection. In this paper we begin a comprehensive study of *M. tuberculosis* gene regulation, characterizing the transcript levels of 10 of its 13 putative sigma factor genes. We developed a real-time RT-PCR assay using a family of novel fluorescent probes called molecular beacons to quantitatively measure the different mRNAs. Three sigma factor genes were identified that have increased mRNA levels after heat shock, two of which also responded to detergent stress. In addition, we also identified a sigma factor gene whose mRNA increased after mild cold shock and a second that responded to conditions of low aeration.

Introduction

Mycobacterium tuberculosis is a slow-growing facultative intracellular parasite. During infection it is exposed to many different environmental conditions depending on the stage and the severity of the disease. It is able to multiply inside the macrophage phagosome, in which the environment is generally hostile for most bacteria. It can also replicate extracellularly in the open lung cavities that are found during the late stages of the disease (Dannenberg and Rook, 1994). *M. tuberculosis* can spread to other tissues or organs such as lymph nodes, bones, joints, skin, the central nervous system, the urinary tract and the abdomen, or it can give a disseminated form of disease (miliary tuberculosis) (Grange, 1996). The host immune response to *M. tuberculosis* initially involves the recruitment of activated macrophages to the site of infection in the lung,

where they can form a tuberculous granuloma that serves to delimit the infection. Bacteria trapped in the granuloma face a hostile environment that becomes anoxic and rich in toxic fatty acids (Dannenberg and Rook, 1994; Grange, 1996). *M. tuberculosis* under these conditions has been postulated to assume a dormant status in which it can remain viable for years without causing observable disease (Wayne, 1994). A subsequent failure of the immune system of the host may permit its emergence from this dormant status, resulting in reactivation of the latent disease. The necessity to adapt to different environments and the possibility of entering a dormant status suggest a major role for the regulation of gene expression in the pathogenicity of *M. tuberculosis*. However, as a result of its slow growth rate and the lack of genetic tools, little is known about gene regulation in this important pathogen.

Alternative sigma factors of bacteria are known to provide a means of regulating gene expression in response to changing environmental stimuli as they can combine with the basic RNA polymerase core enzyme to direct the transcription of new genes. There are many cases of bacterial virulence determinants whose expression is regulated by an alternative sigma factor (Finlay and Falkow, 1997). The most representative examples are the alginate capsule in *Pseudomonas aeruginosa* (Hershberger *et al.*, 1995), ToxR in *Vibrio cholerae* (Parsot and Mekalanos, 1990), some products of the *sar* virulence locus of *Staphylococcus aureus* (Deora *et al.*, 1997) and members of the RpoS regulon in *Salmonella typhimurium* and *Escherichia coli* (Loewen and Hengge-Aronis, 1994).

Recently, the genome sequence of *M. tuberculosis* strain H37Rv has been completed and 13 deduced amino acid sequences have been annotated as putative sigma factors (Gomez *et al.*, 1997; Cole *et al.*, 1998), which all belong to the sigma-70 family (Table 1). Four sigma factors have been partially characterized in various mycobacterial species, but none of them has been functionally characterized in *M. tuberculosis*. *sigA* is presumed to encode the mycobacterial major sigma factor because of its sequence and because its homologue has been shown to be indispensable in *Mycobacterium smegmatis* (Gomez *et al.*, 1998). A *sigA* missense mutation in the region encoding the domain proposed to interact with other regulatory proteins was shown to be responsible for attenuation of virulence in a *Mycobacterium bovis* strain (Collins *et al.*, 1995). *sigB*, closely related to *sigA*, and localized in the same

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Table 1. *M. tuberculosis* sigma factors.

Sigma factor	Class	Function	Reference
σ^A (Rv2703)	Principal sigma factor	Essential in <i>M. smegmatis</i>	Gomez <i>et al.</i> (1998)
σ^B (Rv2710)	Principal sigma factor	A σ^B mutant of <i>M. smegmatis</i> is more sensitive to oxidative stress	Gomez and Smith (manuscript in preparation)
σ^C (Rv2069)	ECF subfamily sigma factor		Cole <i>et al.</i> (1998)
σ^D (Rv3414c)	ECF subfamily sigma factor		Cole <i>et al.</i> (1998)
σ^E (Rv1221)	ECF subfamily sigma factor	A σ^E mutant of <i>M. smegmatis</i> is more sensitive to various stresses	Wu <i>et al.</i> (1997)
σ^F (Rv3286c)	Stress response sporulation sigma factor	Induced during stationary phase, cold shock and various stresses in <i>M. bovis</i> BCG	DeMaio <i>et al.</i> (1996)
σ^G (Rv0182c)	ECF subfamily sigma factor		Cole <i>et al.</i> (1998)
σ^H (Rv3223c)	ECF subfamily sigma factor		Cole <i>et al.</i> (1998)
σ^I (Rv1189)	ECF subfamily sigma factor		Cole <i>et al.</i> (1998)
σ^J (Rv3328c)	ECF subfamily sigma factor		Cole <i>et al.</i> (1998)
σ^K (Rv0445c)	ECF subfamily sigma factor		Cole <i>et al.</i> (1998)
σ^L (Rv0735)	ECF subfamily sigma factor		Cole <i>et al.</i> (1998)
σ^M (Rv3911)	ECF subfamily sigma factor		Cole <i>et al.</i> (1998)

conserved genomic locus in all examined mycobacterial species (Doukhan *et al.*, 1995) is dispensable in *M. smegmatis*. Mutants of *M. smegmatis* with an inactivated *sigB* are more sensitive to hydrogen peroxide and superoxide generating compounds, indicating that σ^B is needed for transcription of genes involved in oxidative stress (M. Gomez and I. Smith, manuscript in preparation). *sigF* encodes a sigma factor related to sporulation-specific sigma factors of *Streptomyces coelicolor* and *Bacillus subtilis*, as well as to the stress-response specific sigma factor σ^B of *B. subtilis* (DeMaio *et al.*, 1997). Interestingly, *sigF* is present only in the slow growing species of mycobacteria. *sigF* expression in *M. bovis* BCG is induced during stationary phase and after exposure to various stresses (DeMaio *et al.*, 1996). *sigE* encodes a sigma factor belonging to a subfamily of sigma factors with similar sequences whose members appear to regulate extracytoplasmic functions (ECFs) and various stress responses (Missiakas and Raina, 1998). *M. smegmatis* strains with an inactivated *sigE* are more sensitive to a variety of stresses such as heat shock, acidic pH, exposure to SDS and oxidative stress (Wu *et al.*, 1997). The other nine putative sigma factors of *M. tuberculosis* H37Rv are classified by their sequence as ECF sigma factors, and have not been characterized (Gomez *et al.*, 1997).

In the present study we developed a real-time quantitative RT-PCR assay that uses a class of novel fluorescent probes, the molecular beacons, to measure the amount of PCR product (Tyagi and Kramer, 1996). Applying this method we studied the transcript levels of the 10 putative sigma factor genes that were known when we began this project. With the recent completion of the H37Rv genome sequence (Cole *et al.*, 1998) another three additional open reading frames have been annotated as putative ECF sigma factor genes. We showed that all of them are

expressed, although in different amounts, during exponential growth. Three sigma factor genes were identified that have increased mRNA levels after heat shock, two of which also responded to detergent stress. In addition, we also identified a sigma factor gene whose mRNA increased after mild cold shock and a second that responded to conditions of low aeration.

Results

Real-time quantitative RT-PCR with molecular beacons

To measure the transcript levels for the sigma factor genes of *M. tuberculosis*, we developed a quantitative RT-PCR assay. In this method, as in traditional RT-PCR, total RNA is used as template to synthesize a cDNA specific for the transcript that is the object of the study. The amount of cDNA produced, which is proportional to the amount of the specific transcript present in the original RNA sample, is measured by quantitative PCR. In the method we have developed the amount of PCR product is determined in real time using molecular beacons. Molecular beacons are hairpin-shaped oligonucleotide probes that consist of a central part homologous to the target, flanked by two 5–6 bp inverted repeats that can form a stable stem. The 5' end of the molecule is coupled to a fluorophore, whereas the 3' end is coupled to a quencher. In the absence of the target the stem is closed and in this conformation the fluorophore is quenched. When molecular beacons bind to their target they undergo a conformational change resulting in the restoration of fluorescence of the internally quenched fluorophore (Tyagi and Kramer, 1996). Molecular beacons are extremely specific probes; in fact they can easily discriminate a target that contains

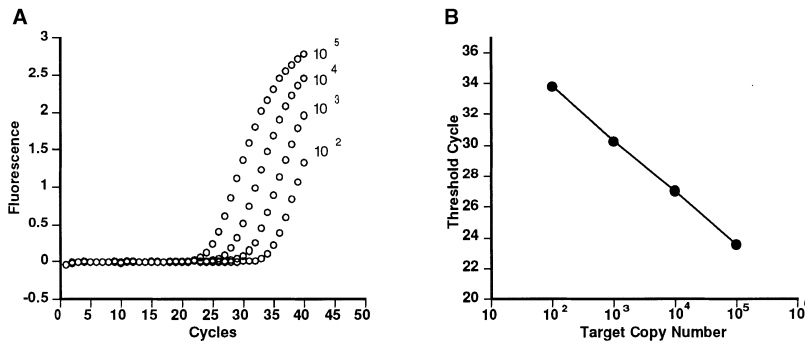


Fig. 1. Real-time measurement of amplicon synthesis during PCR using molecular beacons. Four reactions, each initiated with a different number of template molecules (indicated), were incubated simultaneously in the spectrofluorometric thermal cycler (A). Primers and molecular beacon specific for the *M. tuberculosis sigA* gene were used in the amplification reactions. The target was represented by *M. tuberculosis* H37Rv chromosomal DNA. The amount of chromosome equivalents per μl was calculated considering the length of the *M. tuberculosis* chromosome, 4.4 Mb (Cole *et al.*, 1998) (B). Inverse relationship between the threshold cycle (the cycle at which the fluorescent signal becomes detectable above the background) and the logarithm of the initial number of template molecules. The concentration of amplicons that were present after each cycle of amplification was determined by measuring fluorescence during the last few seconds of the annealing step (Tyagi *et al.*, 1998).

a single mismatch (Marras *et al.*, 1998). When present in a PCR reaction where their target is the amplification product, molecular beacons can form a stable hybrid with it during the annealing step. The intensity of the fluorescence at the annealing step in each amplification cycle is a direct measure of amplicon concentration (Tyagi *et al.*, 1998).

PCR primer pairs and molecular beacons specific for detection of amplification products were designed using the coding sequence of 10 of the 13 putative sigma factors of *M. tuberculosis*. Under the assay conditions the cycle number at which the fluorescence signal become detectable over the background was linear for each primer-beacon set over the range of at least four logs of input DNA molecules (Fig. 1). The same linearity was observed when the template was gene-specific cDNA (data not shown).

Quantification of sigma factor mRNA in exponentially growing *M. tuberculosis*

To estimate the levels of sigma factor gene mRNA present in *M. tuberculosis* during exponential growth, total RNA was extracted from exponentially growing cultures of *M. tuberculosis* H37Rv. Two aliquots of total RNA from independent preparations were subjected to reverse transcription with antisense primers specific for each of the 10 sigma factor genes, and the resulting cDNAs were amplified by PCR in the presence of molecular beacons. Samples containing known amounts of target copies were also used for the molecular beacon analysis in order to provide a standard curve (as shown in Fig. 1) from which mRNA levels were determined. RNA samples not treated with reverse transcriptase were also subjected to PCR in order to measure the level of contamination with chromosomal DNA. For all the genes tested, the samples subjected to

reverse transcription contained a higher amount of template molecules than untreated samples. This demonstrated that all 10 sigma factor genes were transcribed during exponential growth. The most highly represented cDNA was that of *sigC* (5.4×10^5 copies per 10 ng of total RNA), whereas the least represented was that of *sigG* (2.5×10^3 copies per 10 ng of total RNA) (Fig. 2). The cDNA of *sigA*, encoding the putative essential sigma factor, and those of *sigD*, *sigE*, *sigB* and *sigM* were present at levels ranging between 3.7×10^5 and 1.8×10^5 copies per 10 ng of total RNA. The cDNA of *sigF*, *sigH* and *sigI* were present in lower amounts ranging between 3.2×10^4 and 2.7×10^4 copies per 10 ng of total RNA (Fig. 2). As expected, the level of contaminating chromosomal DNA was the same

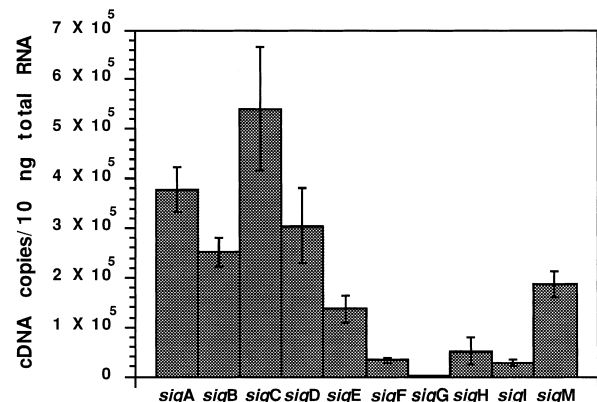


Fig. 2. Levels of various sigma factor mRNAs in exponentially growing cultures. The amount of cDNA copies obtained after reverse transcription of 10 ng of total RNA extracted by exponentially growing *M. tuberculosis* H37Rv, was calculated for each of the 10 sigma factor genes that were the object of this study. The background of contaminating chromosomal DNA (an average of 0.6×10^2 copies per 10 ng of total RNA) was calculated and subtracted. Each measurement was repeated at least twice using independent RNA preparations.

Table 2. Quantification of *sigA* cDNA in *M. tuberculosis*.

Culture condition	<i>sigA</i> cDNA copies per 10 ng total RNA
Exponential phase	378 134 ± 22%
1 mM H ₂ O ₂	336 921 ± 15%
10 mM H ₂ O ₂	333 576 ± 10%
pH 5.0	312 995 ± 7%
0.05% SDS	360 255 ± 20%
Room temperature	343 275 ± 10%
Ice bath	307 749 ± 12%
45°C	331 015 ± 14%
Standing culture	132 300 ± 14%
Stationary phase	116 802 ± 20%
H ₂ O	96 012 ± 22%

Each measurement was repeated at least twice using independent RNA preparations.

in all the reactions (an average of 6×10^2 copies per 10 ng of total RNA). This represented (with the exception of *sigG*) between 1/60 and 1/900 of the amount of the detected cDNA.

Amount of *sigA* mRNA under different stress conditions

To study the levels of mRNAs for various genes in cultures subjected to various stress conditions by quantitative RT-PCR, it is useful to have as an internal standard a gene whose mRNA levels do not change during exposure to these conditions. As the *sigA* product is presumed to be the essential housekeeping sigma factor of *M. tuberculosis* (Gomez *et al.*, 1998), its mRNA was considered a good candidate for such an internal control. To test this hypothesis, we measured the amount of *sigA* mRNA in RNA preparations from *M. tuberculosis* H37Rv cultures that had been exposed to various stresses that have previously been used in mycobacterial species (DeMaio *et al.*, 1996; Wu *et al.*, 1997) (described in *Experimental procedures*), as well as from an H37Rv culture grown to stationary phase and one incubated 24 h without shaking (low aeration). The results, shown in Table 2, indicate that the amount of *sigA* transcript was practically the same in all the conditions tested, with the exception of incubation in water, growth into stationary phase and growth in the absence of shaking. In these cases the amount of *sigA* transcript was 3.3-, 3.0- and 3.9-fold less than in total RNA preparations obtained from bacteria grown to mid-exponential phase.

Changes in sigma factor mRNA levels after exposure to different stress conditions

As the amount of *sigA* mRNA was constant in 8 of the 11 conditions tested, it was used as an internal standard in relative RT-PCR assays to quantify the changes in the

level of the mRNAs specific for the different sigma factors in these conditions. Equal amounts of H37Rv total RNA extracted from a culture grown to mid-exponential phase and from cultures exposed to the different stress conditions were subjected to reverse transcription with antisense primers specific for *sigA*, the internal standard, as well as with antisense primers specific for the other sigma factor genes. The reverse transcriptions were carried out in the same tube in order to prevent sample to sample variability. After reverse transcription, the amount of cDNA obtained for each transcript was measured by PCR with molecular beacons. The amount of contaminating chromosomal DNA was tested in parallel samples not treated with reverse transcriptase and the values obtained were used to correct the values obtained after reverse transcription. The amount of mRNA for each sigma factor gene was calculated normalizing for the amount of *sigA* transcript. No changes in the amount of any sigma factor mRNA were found in RNA preparations from bacteria exposed for 2 h either to H₂O₂ (1 mM or 10 mM), to pH 5.0, or after incubation in an ice bath (data not shown). However, major differences were found in the level of sigma factor mRNAs in RNA preparations obtained from bacteria exposed to 0.05% SDS, heat shock (45°C) and mild cold shock (room temperature). After exposure to SDS, the levels of *sigB* and *sigE* transcripts increased 11- and 6-fold, respectively, whereas the level of *sigC* and *sigM* transcripts decreased 7- and 3.5-fold respectively (Fig. 3A). After exposure to heat shock the levels of *sigB*, *sigE* and *sigH* transcripts rose 23-, 3- and 20-fold, respectively, whereas the levels of *sigC* and *sigD* transcripts decreased 9- and 5-fold respectively (Fig. 3B). After exposure to mild cold shock, the transcript levels that showed the highest variation were those of *sigI*, which rose 3-fold and *sigG*, which decreased 3.4-fold (Fig. 3C). The amount of mRNA for each sigma factor gene was also calculated by normalizing for the total amount of RNA used as starting material. The results were essentially the same as those obtained by normalizing for *sigA* transcripts (data not shown).

We calculated the levels of the sigma factor gene transcripts under conditions in which the *sigA* transcript was not constant (H₂O, stationary phase, low aeration) by normalizing for the total amount of RNA used as starting material. After incubation of bacteria in H₂O for 2 h, almost all the sigma factor transcripts decreased in amount, although to various extents. The only exception was the *sigI* transcript which remained constant (Fig. 3D). The amount of all sigma factor gene transcripts obtained from cells grown in standing culture or grown to stationary phase decreased, with the exception of the *sigB*, *sigE* and *sigF* transcripts. These remained constant in these conditions, except the *sigB* transcript, which increased 2.5-fold in cells grown in low aeration (Fig. 3E and F).

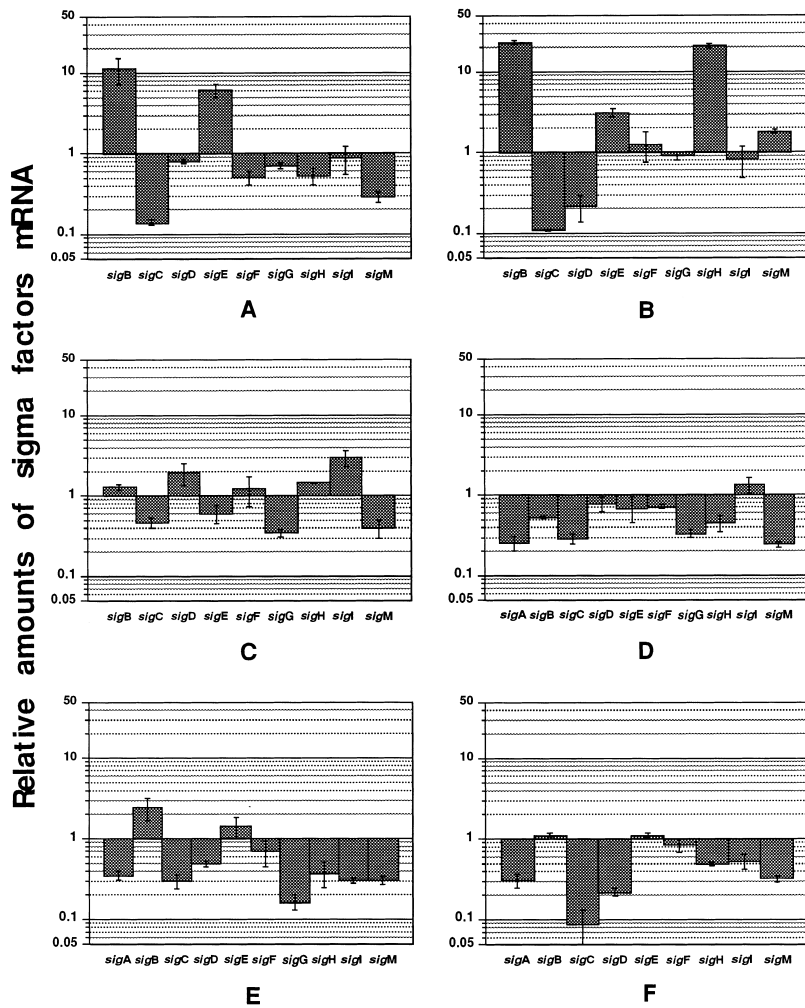


Fig. 3. Changes in sigma factor mRNA levels after exposure to different stresses. The values are expressed as the ratio between the number of cDNA copies detected in samples obtained from the stressed culture, and the number of cDNA copies detected in samples obtained from exponentially growing bacteria. For the stress conditions where the level of *sigA* transcript was invariable (exposure to SDS, heat shock, and mild cold shock), the values were normalized to *sigA*. For the stress condition where the level of *sigA* transcript varied (exposure to H₂O, low aeration and stationary phase), values were normalized to the amount of RNA used as starting material.

A. Exposure to SDS.
 B. Heat shock.
 C. Mild cold shock (room temperature).
 D. Exposure to H₂O.
 E. Low aeration.

F. Stationary phase. *sigG* was not included in F because its transcript was undetectable over the background.

Each measurement was repeated at least twice using independent RNA preparations.

Discussion

We analysed the mRNA levels of 10 sigma factor genes of *M. tuberculosis* H37Rv under various stresses and culture conditions. For this purpose we developed a real-time relative RT-PCR assay that uses a family of novel fluorescent probes, the molecular beacons, to detect and measure the amount of PCR product. Recently, different classes of fluorescent probes used in combination with the ABI 7700 Prism spectrofluorometric thermal cycler have been shown to be rapid and sensitive alternatives to competitive PCR (Desjardin *et al.*, 1998; Tyagi *et al.*, 1998). In these systems, the amplification of the PCR product is followed in real time by measuring the fluorescence produced by the probe in the presence of its target. These methods are less labour intensive than competitive RT-PCR, and reduce the risk of template contamination because no post-amplification handling is required. The amount of target DNA in a given sample is interpolated from a standard curve run in parallel with the unknown samples. Using this method and

normalizing for the amount of total RNA used as starting material, we first showed that mRNAs of the 10 sigma factor genes in this study are present during exponential growth, although in different amounts. The mRNA of *sigC* was the most abundant (5.4×10^5 copies per 10 ng of total RNA). This finding is surprising, because we know from previous data that during exponential growth of *M. tuberculosis* most of the RNA polymerase molecules are associated either with σ^A or σ^B (unpublished data). It is possible that the *sigC* transcript is translated at a very low efficiency, that σ^C is unstable or that it has a low affinity with the RNA polymerase. A problem that should also be considered when RT-PCR analysis is used to measure the absolute amount of different transcripts, is that a variation in the efficiency of reverse transcription of different transcripts could influence the results.

These experiments showed that all 10 sigma factor genes we analysed were expressed in the exponential phase of growth. We then wanted to study their mRNA levels under different stress and growth conditions. We

first compared the levels of *sigA* transcript from cultures growing in exponential phase and after exposure to different stresses and growth conditions. In agreement with the hypothesis that *sigA* encodes the major vegetative sigma factor of *M. tuberculosis* (Gomez *et al.*, 1998), we found that *sigA* transcript was present at equal levels under all the conditions tested except after exposure to H₂O, growth in low aeration and growth to stationary phase. In these conditions the level of *sigA* mRNA decreased about 3-fold. The energy available to the bacteria under these conditions may be low, and it is possible that the decreased levels of the *sigA* transcript reflect a decrease in the mRNA pool relative to the total RNA rather than a specific down regulation of the *sigA* gene. Based upon these observations, we used the *sigA* transcript as an internal standard to calculate the transcript levels for the other sigma factor genes only under those conditions where its level was invariant. Using this method we showed a dramatic increase in the level of *sigB* and *sigH* transcripts as well as an appreciable increase in the level of *sigE* transcript in response to heat shock. In the same conditions the *sigD* transcript decreased significantly. The level of *sigB* and *sigE* mRNAs increased dramatically in response to the presence of the detergent SDS in the culture medium. Interestingly, under both these conditions the *sigC* transcript, the most abundant during exponential phase, decreased ≈ 10 -fold.

Proteins of the heat shock regulon have been shown to be important for virulence in many bacterial pathogens. These include proteins implicated in general stress response, such as GrsA in *Yersinia enterocolitica* (Yamamoto *et al.*, 1997), or HtrA in *Salmonella* (Sinha *et al.*, 1997) and *Brucella* (Elzer *et al.*, 1996), as well as proteins whose role is specific to the pathogenic mechanism of the bacterium (i.e. the enzymes of the biosynthetic pathway for the alginate capsule in *P. aeruginosa* (Schurr and Deretic, 1997) and a protein of *Helicobacter pylori* required for adhesion to the gastric epithelium (Yamaguchi *et al.*, 1997). The identification of three heat shock responsive sigma factor genes in *M. tuberculosis* is the first step in a comprehensive study of the possible involvement of their regulons in the pathogenic process. Two of the three heat shock sigma factor gene transcripts (*sigB* and *sigE*) were also present at higher levels after exposure to SDS, which suggests that the signal inducing these changes could be extracellular (e.g. surface protein denaturation, etc.). The higher response of *sigE* and the lower response of *sigB* after exposure to SDS than after heat shock suggest some difference in the modulation of the signal between the two stresses. On the other hand the fact that the *sigH* transcript increased during heat shock, but not during SDS treatment, suggests that the signal in this case could be intracellular (i.e. the denaturation of cytoplasmic proteins).

Mycobacteria are resistant to high concentrations of SDS: the finding of a stress response to SDS treatment in *M. tuberculosis*, together with the fact that a *sigE* mutant of *M. smegmatis* is more sensitive to this detergent (Wu *et al.*, 1997), suggest the existence of a specific mechanism of resistance in addition to that due to the peculiar structure of the lipid rich mycobacterial cell wall. It is worth noting that the first environment encountered by *M. tuberculosis* inside the host is the internal part of the alveoli that is coated with pulmonary surfactant. This is a mixture of many molecular species, mainly phospholipids and specific proteins involved both in the organization of the surfactant phospholipids and in innate host defence functions (Johansson and Curstedt, 1997). It is possible that this detergent-like material could produce a stimulus similar to that of SDS and induce the increase of *sigB* and *sigE* transcripts. If this is confirmed experimentally, it would suggest the *M. tuberculosis* adaptation to the alveolar environment as a physiological basis for the observed SDS response.

Exposure of bacteria to mild cold shock (room temperature) for 2 h produced a 3-fold increase in the transcript level for *sigI*. *M. tuberculosis* is not believed to have a host-free stage in its life cycle. However, before infecting a new host *M. tuberculosis* spends some time in aerosol particles where the ambient temperature is usually lower than 37°C. The fact that the transcript level of *sigI* increases after mild cold shock suggests that its regulon could be involved in the survival of *M. tuberculosis* during this unknown and important stage of its life cycle.

After exposure for 2 h to oxidative stress, acid stress or cold shock no significant variation of the transcript level for any sigma factor gene was observed. This is in contrast with a previous finding that in *M. bovis* BCG the *sigF* mRNA level increases 4.8 and 17.6 times after oxidative stress and cold shock respectively (DeMaio *et al.*, 1996). In those cases bacteria were exposed to the stress conditions for 24 h. As we believe that a specific stress response needs to be fast in order to be effective, we used a shorter period of exposure to the stress conditions (2 h), and this may explain the differences. Furthermore *sigF* may be regulated differently in *M. tuberculosis* than in *M. bovis* BCG.

The relative amounts of sigma factor mRNAs were obtained after normalizing to an invariable internal standard (i.e. the *sigA* transcript). This method is preferable in quantitative RT-PCR, because it corrects the sample to sample variability sometimes observed in reverse transcription, and eliminates the requirement for an accurate measurement of total RNA (allowing for analysis of small amounts of RNA, as required to study transcript levels in infected tissues). Nonetheless, when no internal standard can be found, as in case of cultures exposed to H₂O, grown in low aeration and grown to stationary phase, the amount of total RNA used as starting material can be used as normalization (Nakayama *et al.*, 1992). Using this method we

measured the level of the transcript for 10 sigma factor genes in these three stress conditions. We found a general decrease in the level of all sigma factors transcripts except that of *sigI* after exposure to H₂O, and those of *sigB* and *sigE* in cultures grown with low aeration or to stationary phase. The level of the *sigF* transcript was constant in cultures grown to stationary phase. The general decrease of almost all the tested transcripts reinforces the hypothesis that in these growth conditions, probably characterized by low available energy, the mRNA pool drops with respect to the total RNA.

Biochemical data suggest that *M. tuberculosis* growing *in vivo* shifts to anaerobic metabolic pathways (Segal, 1984). Moreover it has been shown that tubercle bacilli are able to differentiate into a non-replicating stage when the oxygen is depleted from the medium at a slow rate. This ability has been hypothesized to be responsible for the capability of *M. tuberculosis* to lie dormant in the host for long periods of time (Wayne and Hayes, 1996). The finding that the *sigB* transcript increases 2.5 fold under conditions of low aeration, when the levels of the other sigma gene transcripts drop, suggests an involvement of the σ^B regulon in the response to oxygen depletion. We found no increases in the mRNA levels of the 10 sigma factor genes after 70 h in stationary phase. Only the *sigB*, *sigE* and *sigF* transcripts remained constant suggesting that their regulons may be important at this stage. Our different results from previously published data showing that *sigF* is induced both in stationary phase and in conditions of low aeration in *M. bovis* BCG (DeMaio *et al.*, 1996) suggests a different regulation of this gene in *M. tuberculosis*.

In this study we began a comprehensive characterization of the differential expression of 10 sigma factor genes of *M. tuberculosis*. We identified three heat shock responsive sigma factor genes, *sigB*, *sigH* and *sigE*, of which *sigB* and *sigE* also responded to detergent stress and a mild cold shock responsive sigma factor gene, *sigI*. We showed also that *sigB* responds to conditions of low aeration and that the level of some transcripts, especially that of *sigC*, dramatically decreases under specific conditions. Of course, the detection of a variation in the mRNA level of a gene under particular conditions does not necessarily indicate the levels of the translated protein or the gene function. However, this information can suggest the design of other, more specific experiments to understand the biology of the gene and its product.

The sensitivity and versatility of the quantitative RT-PCR assay with molecular beacons described in this paper will enable us to study the gene expression of *M. tuberculosis* in cultured macrophages and in infected tissues, where only small amounts of mycobacterial RNA are available. We are currently analysing the role of the alternative sigma factors in *M. tuberculosis* pathogenesis by studying their expression *in vivo* (both in human macrophages and

in guinea-pig granulomas), and by producing strains with mutated sigma factor genes to test their virulence.

Experimental procedures

Bacterial strains, media and growth conditions

M. tuberculosis strain H37Rv cultures were grown in roller bottles at 37°C in Middlebrook 7H9 liquid medium (Difco) supplemented with 10% bovine serum albumin fraction V, dextrose and sodium chloride (ADC) (Difco), 0.2% glycerol and 0.05% Tween-80. Treatment of the bacteria with the various stress conditions was as follows: an exponentially growing culture of H37Rv was divided into 10 ml aliquots, bacteria were centrifuged at 3000 × *g* for 4 min at room temperature and each aliquot was resuspended in 10 ml of the appropriate stress medium (pre-equilibrated at the temperature to be used in the stress experiment), and subjected to the stress for 2 h. The stress conditions were: 1 or 10 mM H₂O₂ (oxidative stress); low pH (pH 5.0) (acid stress); 0.05% SDS (detergent stress); H₂O (hypo-osmotic shock); ice bath (cold shock); room temperature (mild cold shock); 45°C (heat shock). With the exception of cold and heat shock, bacteria were incubated in roller bottles during the exposure to the stress conditions. For exposure to low aeration, the culture was incubated for 24 h at 37°C without agitation. For stationary phase, bacteria were collected at OD₆₀₀ 2.8 (10 mm cell; Spectronic 21D spectrophotometer, Milton Roy). After being subjected to the stress conditions, bacteria were 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). Finally, the bacterial pellets were frozen on dry ice and stored at -70°C.

RNA extraction

Bacteria were thawed on ice and added to prechilled 15 ml screw-capped polypropylene centrifuge tubes containing 1 ml of zirconia/silica beads (0.1 mm diameter, Biospec Products) and 1 ml of phenol-chloroform-isoamyl alcohol (25:24:1). The tubes were shaken in a vortex mixer at the maximal speed for 4 min, in 1 min pulses followed by chilling in an ice bath. Samples were then centrifuged at 3000 × *g* for 5 min at 4°C, and the upper phase was transferred to another prechilled tube containing 1 ml of phenol-chloroform-isoamyl alcohol and the extraction was repeated. To the upper phase of each sample 1/10 Vol. of 2 M LiCl and 2.5 vols of ethanol were added, and after an incubation of 10 min in an ice bath samples were centrifuged at 10 000 × *g* for 10 min at room temperature. After centrifugation the pellet was washed with 75% ethanol, dried, and resuspended in 100 µl of diethylpyrocarbonate (DEPC)-treated dH₂O. To remove the chromosomal DNA, the samples were treated three times with TRI Reagent (Molecular Research Center) according to the manufacturer's instructions. The sample (100 µl) was added to 1 ml TRI reagent and mixed. After incubation at room temperature for 5 min, 0.2 ml of chloroform were added and after mixing carefully the sample was again incubated for 5 min at room temperature. The samples were centrifuged at 10 000 × *g* for 10 min at 4°C, and the upper phase was added to 0.5 ml of isopropanol. After incubation

Table 3. Primers used to amplify the internal fragments of the sigma factor genes. The antisense primers were also used to prime cDNA synthesis.

Gene	Forward primer ^a	Antisense primer	PCR product (bp)
<i>sigA</i>	5'-GAGATCGGCCAGGTCTACGGCGTG-3'	5'-CTGACATGGGGGCCCGCTACGTTG-3'	160
<i>sigB</i>	5'-CGCCGCGGGTGAAGTCGAA-3'	5'-CAGGTCGCGTTTTCGGTTCT-3'	106
<i>sigC</i>	5'-CGCGCGTCCCGAACATCTCA-3'	5'-GTCGGCGATCATCGTGGTTAC-3'	85
<i>sigD</i>	5'-CGGCCCTGTACGAGATGACG-3'	5'-CCGCGATGCCGTACAGAAAC-3'	113
<i>sigE</i>	5'-GTTGCCGACGGTGACGACTTG-3'	5'-CGCGGACCTGTTGGGGATGAG-3'	115
<i>sigF</i>	5'-GCGGGTCGGGCTGGTCAAC-3'	5'-CCTCGCCCATGATGGTAGGAAC-3'	92
<i>sigG</i>	5'-CTGCGTAGGCTCATTGACGTG-3'	5'-CGGTGTGGGCGGAGAAGTC-3'	91
<i>sigH</i>	5'-GGCAACCGGCGGAGATCC-3'	5'-GCAGCCCGGTCGAGGAATG-3'	81
<i>sigI</i>	5'-CGCATGGCACGCGGTGAC-3'	5'-AGCAAAGCCAGCCGGACCTC-3'	87
<i>sigM</i>	5'-ACCGCGCAGGTCGAGACC-3'	5'-GGGTGTCGGCGATCGAATAG-3'	115

a. The co-ordinates of the primers are available upon request.

at room temperature for 10 min the samples were centrifuged at 10 000 × *g* for 15 min at 4°C. Pellets were washed three times with 75% ethanol, dried, and resuspended in 100 µl of DEPC-treated dH₂O. RNA quantification was carried out both by spectrophotometry, and fluorometry with SYBR Green (Molecular probes); each sample was diluted to a concentration of 250 ng µl⁻¹ and frozen in aliquots at -70°C. Five-hundred nanograms of RNA were separated by agarose gel electrophoresis and visualized by ethidium bromide staining to verify the quality of the samples. RNA was deemed acceptable for further use if the amount of 23s rRNA was twice that of 16s rRNA.

PCR primers and design of molecular beacons

All PCR primers, listed in Table 3 with their amplification products, were designed to anneal to their target at the same temperatures (57°C) and to amplify DNA fragments internal to the coding sequence of the relevant genes. Ten molecular beacons were designed, each able to hybridize to one of the 10 PCR products (Table 4). To ensure that all molecular beacons can work in the same conditions the following criteria should be met at the detection temperature (the annealing step of the PCR reaction, 57°C): in the absence of target, the hairpin stem must be closed; in the presence of the target, the molecular beacon must form a stable probe-target hybrid. Using a DNA folding program to estimate the stability of the hairpin stem (available at the internet address <http://www.ibc.wustl.edu/~zucker/dna/form1.cgi>), we chose hairpin stems

that dissociate at a temperature 7–8°C higher than the 57°C detection temperature. Using the 'percentage-G:C rule' (Lathe, 1985), we chose probe sequences that resulted in probe-target hybrids that dissociate at a temperature 7–8°C higher than the detection temperature (Marras *et al.*, 1998).

Synthesis of molecular beacons

Molecular beacons were synthesized from oligonucleotides containing a primary amino group at their 3' end and a sulphhydryl group protected by a trityl moiety at their 5' end (Midland Certified Reagents) as previously described (Tyagi and Kramer, 1996). Briefly, an amino-reactive derivative of DABCYL ((4-(4'-dimethylaminophenylazo)benzoic acid)succinimidyl ester) (Molecular probes), was covalently linked to the 3' amino group. After coupling, the oligonucleotides were purified by high-pressure liquid chromatography. The protective trityl moiety was then removed from the 5' sulphhydryl group, which was then coupled with an iodoacetamide derivative of fluorescein (5-iodoacetamidofluorescein) (Molecular Probes). Finally, the oligonucleotides containing both modifications were purified by high-pressure liquid chromatography. A detailed protocol for synthesizing molecular beacons is available at the following internet address: http://www.phri.nyu.edu/molecular_beacons.

Reverse transcription

For each sample, 500 ng of total RNA (2 µl) was added to

Table 4. Sequence of the molecular beacons used in this study.

Gene	Molecular beacons ^a
<i>sigA</i>	Fluorescein-5'- <u>CCGAG</u> AGTTGCGCCATCCGA <u>CTCGC</u> -3'-DABCYL
<i>sigB</i>	Fluorescein-5'- <u>CCACGG</u> AAGCGCATAGAAGCCGGG <u>CCGTGG</u> -3'-DABCYL
<i>sigC</i>	Fluorescein-5'- <u>CCACGC</u> CCATGCCCGCGGATTTCGAA <u>CCGTGG</u> -3'-DABCYL
<i>sigD</i>	Fluorescein-5'- <u>CCTGGG</u> ACCATAACGGCGCTGCCGC <u>CCCAGG</u> -3'-DABCYL
<i>sigE</i>	Fluorescein-5'- <u>CCAGGG</u> ATCAGACCATCACGACCTTG <u>CCCTGG</u> -3'-DABCYL
<i>sigF</i>	Fluorescein-5'- <u>GGGCCT</u> TCGGACTTCGTCTCCTTC <u>AGGCC</u> -3'-DABCYL
<i>sigG</i>	Fluorescein-5'- <u>GGGTCC</u> CGGGCTCGCGCCAGAGT <u>GGACCC</u> -3'-DABCYL
<i>sigH</i>	Fluorescein-5'- <u>CGCTCG</u> CGATTGGCAACTGGCGTCC <u>CGAGCG</u> -3'-DABCYL
<i>sigI</i>	Fluorescein-5'- <u>CCAGGC</u> AGCCGGTCAACCGATGAC <u>CCCTGG</u> -3'-DABCYL
<i>sigM</i>	Fluorescein-5'- <u>CCAGGC</u> CACTGATGCGGCTGCCCGT <u>GCCTGG</u> -3'-DABCYL

a. The co-ordinates of the beacons are available upon request. Underlined sequences represent the inverted repeated arms of the molecular beacons.

25 pmol of each antisense primer (up to five different primers were used in the same annealing mixture) and 2 μ l of 5 \times Avian Myeloblastoma Virus Retro-Transcriptase (AMV) buffer (USB) in a volume of 10 μ l. After denaturation at 95°C for 1 min and 30 s, the annealing between the RNA and the antisense primers was carried out for 2 min at 50°C. Subsequently, 3.5 μ l of the annealing mixture were added to 1.1 μ l of dNTP mixture (1 mM each), 0.4 μ l of 5 \times AMV RT buffer, and 8 units of AMV (USB) in a final volume of 5.5 μ l. Samples were incubated for 40 min at 47°C, heated at 95°C for 1 min, and then chilled on ice. Identical samples not treated with AMV were prepared as a control. Samples were then diluted with 70 μ l of H₂O and stored at -70°C.

PCR with molecular beacons

PCR conditions were identical for all reactions. The 50 μ l reactions consisted of 1 \times TaqMan Buffer A (Perkin-Elmer), 4 mM MgCl₂, 0.25 mM each dNTP, 2.5 units of AmpliTaq Gold polymerase (Perkin-Elmer), 0.5 μ M of each primer, and 0.3 μ M of the appropriate molecular beacon. Forty cycles of amplification (94°C denaturation for 30 s, 57°C annealing for 60 s, and 72°C polymerization for 30 s) were carried out in sealed tubes in an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler (Perkin-Elmer). Fluorescence was measured during the annealing step and plotted automatically for each sample. For RT-PCR, 3.5 μ l aliquots of the diluted reverse transcription reaction were used as template (equivalent to 8 ng of total RNA used as starting material).

Quantitative analysis of the data

It has been shown that the threshold cycle of a PCR with molecular beacons (the cycle at which the fluorescence becomes detectable above the background) is inversely proportional to the logarithm of the initial number of template molecules (Tyagi *et al.*, 1998). From the threshold cycle obtained amplifying known amounts of target, it is possible to calculate an equation of the type: $n = a + b \log(x)$, where n is the number of target copies, x is the threshold cycle obtained amplifying n copies, a is the intercept and b is the slope. Applying this equation is possible to extrapolate the amount of target copies contained in an unknown sample amplified in a parallel reaction. For each PCR primer-beacon set, PCR was performed in parallel reactions using different amounts of H37Rv chromosomal DNA (100, 1000, 10 000 and 100 000 theoretical copies) (Fig. 1), and two different cDNA preparations, each one obtained from an independently prepared total RNA (10 ng). RNA samples not previously subjected to reverse transcription were also amplified to measure the amount of contaminating chromosomal DNA. The constants (a and b) for each PCR primer-beacon set were calculated, and the resulting equation was used to estimate the number of cDNA copies present in the uncharacterized samples.

In the case where the amount of *sigA* transcript was constant we calculated the relative change in mRNA copies of the other *sig* genes from cultures subjected to various stress conditions, by normalizing to the amount of *sigA* mRNA. The reverse transcription of the mRNAs of *sigA* and up to four other genes was carried out in the same tube in order to prevent sample to sample variability. After reverse transcription,

the amount of cDNA obtained from each transcript was measured by PCR with molecular beacons in a separate reaction. The amount of contaminating chromosomal DNA was tested in parallel samples not treated with reverse transcriptase. The change of specific mRNA levels after exposure of the culture to the stress condition was calculated as the ratio between the number of *sigA* cDNA copies and the number of cDNA copies of the gene of interest in a given RNA preparation divided by the same ratio in an RNA preparation obtained from a mid-log culture. For each sample the amount of contaminating DNA was calculated and subtracted.

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