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Differential Expression of *sigH* Paralogs during Growth and under Different Stress Conditions in *Mycobacterium smegmatis*▽†

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SigH regulates a transcriptional network that responds to heat and oxidative stress in mycobacteria. Seven *sigH* paralogs are reported to exist in the *Mycobacterium smegmatis* genome. A comprehensive real-time reverse transcriptase PCR analysis during different stages of growth and upon exposure to various stress conditions and antimycobacterial compounds showed differential expression of *sigH* paralogs during stationary phase and severalfold increases in the levels of transcription of *sigH1*, *sigH4*, *sigH5*, *sigH6*, and *sigH7* under specific stress conditions.

Sigma factors play a major role in the regulation of bacterial gene expression, and their contents vary considerably in different mycobacterial genomes (18). The recently sequenced *Mycobacterium smegmatis* genome is predicted to encode 26 sigma factors, which is twice the number present in *Mycobacterium tuberculosis* (13 sigma factors) (23). *M. smegmatis* contains orthologs of all *M. tuberculosis* sigma factors except *sigC*, *sigI*, and *sigK*. There is an enrichment of the *sigH* subfamily in this species that contains seven paralogs (23). SigH, an extracytoplasmic function (ECF) sigma factor, is a key regulator of a transcriptional network that responds to oxidative and heat stresses in mycobacteria (17). *M. smegmatis* and *M. tuberculosis* SigH are highly similar proteins, and their levels are found to be increased by heat shock and oxidative stress in both species. However, an *M. tuberculosis sigH* mutant is more susceptible to heat and oxidative stress. An *M. smegmatis sigH* mutant showed survival at 53°C, like the wild type, but increased sensitivity toward cumene hydroperoxide (5). Similar survival instincts of the *sigH* mutant and the wild type suggest the presence of multiple mechanisms to counter these stresses in this species. Although the regulatory mechanism of *sigH* expression and *sigH*'s role have been worked out (13, 16, 22), the multiplicity of its paralogous circuit in *M. smegmatis* remains to be analyzed. Determining the conditions under which the expression of *sigH* paralogs are induced or repressed is vital to the understanding of their possible function and role in the regulation of gene expression in *M. smegmatis* in response to different environmental stimuli. We examined the expression of *sigH* paralogs in *M. smegmatis* at different stages of growth and upon exposure to various stress conditions, like heat shock, cold shock, nutrient starvation, oxidative stress, and antibiotic stress, using quantitative real-time reverse transcriptase PCR.

Organization of *sigH* paralogs in the *M. smegmatis* genome. ECF sigma factors are known to exist as operons in several bacterial genomes (8, 24). Of the eight *sigH* subfamily members, present at different loci in the *M. smegmatis* genome, six are clustered in putative operons, while *sigH2* and *sigH4* are monocistronic (Fig. 1). *sigH* overlaps *rshA*, a gene encoding *sigH*'s cognate anti-sigma factor. The *sigH1* and *sigH3* operons include four genes; *sigH1* is the third gene in its operon and is followed by a gene encoding a putative transcriptional regulator with cupin domains (MSMEG_3484), while *sigH3* is the last gene in its operon and is preceded by *usfY* (MSMEG_4406) and the MSMEG_4408 gene, both of which are predicted to encode membrane proteins. The monocistronic units *sigH2* (MSMEG_0573) and *sigH4* (MSMEG_0574) are present on complementary strands; *sigH2* is followed by genes encoding a series of hypothetical proteins, and *sigH4* is followed by genes encoding two mycobacterial transmembrane proteins, Mmps1 (MSMEG_0575) and MmpL4 (MSMEG_0576). *sigH5* is preceded by a transcriptional regulatory protein (MSMEG_1691) in a putative eight-gene operon and followed by an oxoacyl reductase (MSMEG_3484) and a cupin domain protein (MSMEG_3484). *sigH6* is the last gene of a putative tricistronic operon; it is preceded by two genes encoding hypothetical proteins and followed by a gene encoding a transcriptional regulator protein (MSMEG_3297) after a gap consisting of a 175-bp intergenic region. *sigH7* is the first gene in a putative tricistronic operon and overlaps a gene encoding a cupin domain protein (MSMEG_1748).

Expression of *sigH* paralogs during growth and stress conditions. *M. smegmatis* strain mc^2155 was grown at 37°C in 7H9 medium, and 10-ml cultures were removed at timely intervals of 12 h, 24 h, 48 h, and 72 h and processed for RNA isolation, as described previously (20). For stress experiments, aliquots of exponentially growing cultures were subjected to various treatments for 4 h: cold shock and heat shock (15°C and 53°C, respectively), nutrient starvation (phosphate-buffered saline, pH 7.0), and oxidative stress (10 mM H_2O_2). For antibiotic treatments, compounds at their high-end critical concentrations, namely, isoniazid (200 μg/ml), rifampin (rifampicin) (200 μg/ml), ethambutol (5 μg/ml), and streptomycin (2 μg/ml) (20), were added to the culture, which was incubated for 4 h

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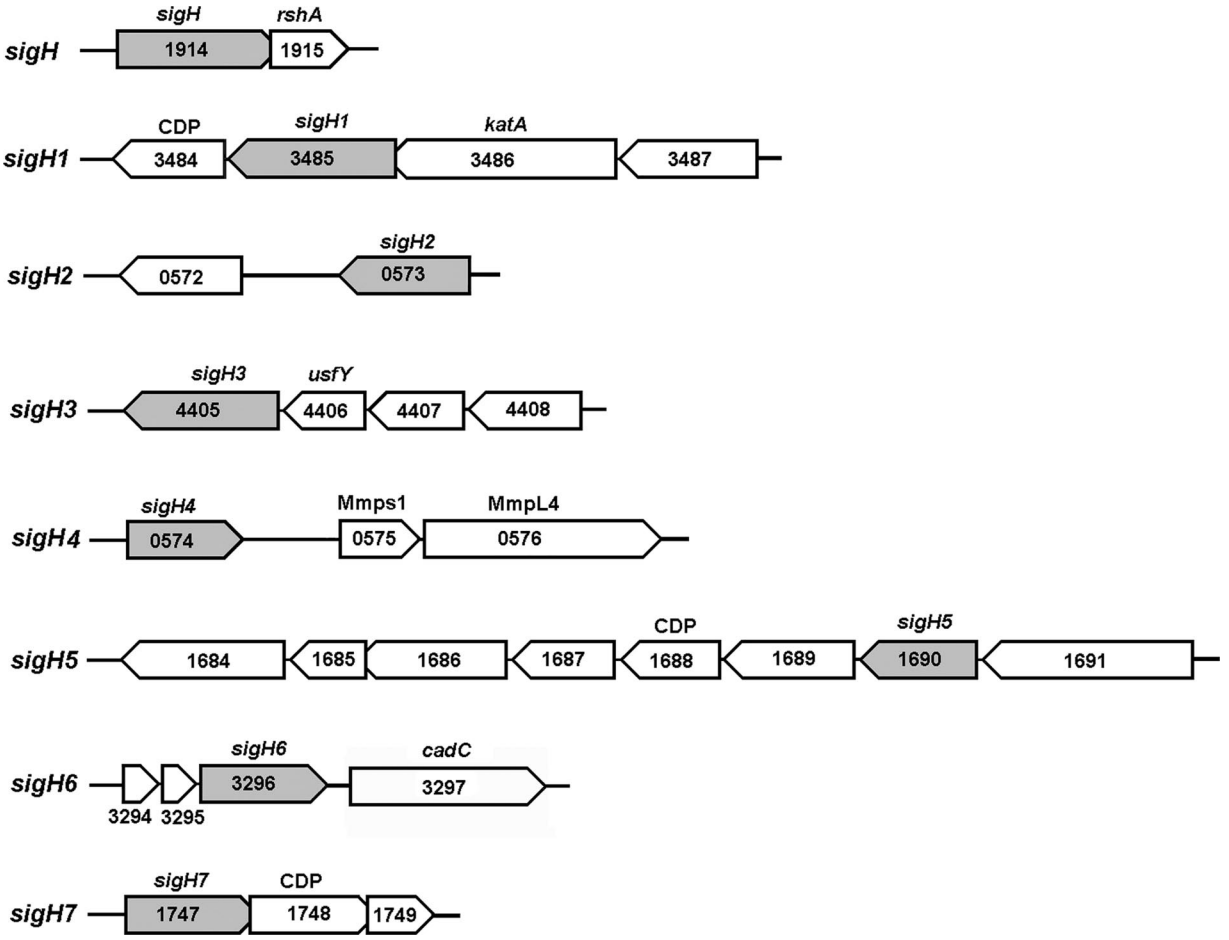

FIG. 1. Genomic organization of *sigH* paralogs in *M. smegmatis*. Sigma factors other than *sigH2* and *sigH4* are arranged in a polycistronic operon. Open reading frames encoding cupin domain proteins (CDP) and putative membrane proteins follow *sigH* paralogs in the putative operon. Blunt-tipped arrowheads indicate the overlap of open reading frames.

and then processed for RNA isolation. About 5.0 μg of DNA-free RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase, and cDNA was purified, diluted five times with sterile water, and processed for quantitative real-time PCR using appropriate primers of *sigH* paralogs (see Table S1 in the supplemental material) and Roche's SYBR green I master kit on a model 480 LightCycler (Roche Diagnostics). Melt curve analysis was performed to ensure the homogeneity of each amplicon. Relative expression levels were determined after normalization with the *sigA* transcript level using LightCycler 480 II software version 1.5 (Roche, Germany). The average relative expression levels and standard deviations were determined from the data generated from three independent experiments.

First, we examined the expression of *sigH* paralogs from the early exponential phase to the late stationary phase of growth. Figure 2 shows the relative expression levels of *sigH* paralogs at the various phases of growth. The transcript level of *sigH* increased marginally during late log phase, declined to nearly the control level in stationary phase, and then increased to 2.6-fold during late stationary phase. *sigH1* expression remained relatively constant during the early and late log phases but gradually increased to 8.3-fold and 10.4-fold during the stationary and late stationary phases, respectively. *sigH2* showed a gradual increase of 6.6-fold during log phase, an increase of 16.6-fold during stationary phase, and then a decrease to 7.3-fold in late stationary phase. *sigH3* and *sigH5* levels were induced to 4.7-fold and 3.2-fold, respectively, during stationary phase but declined to much lower levels during late stationary phase. *sigH4* did not respond to stationary phase. *sigH6* and *sigH7* transcripts were far below the control levels during these stages. An *M. smegmatis sigH* mutant displayed survival in the logarithmic and stationary phases of growth similar to that of the wild type (5). The lower level of *sigH* expression indicates the lesser dependence of *M. smegmatis* on *sigH*-mediated gene expression during different stages of growth. Further, it is possible that the parallel expression of *sigH* paralogs would have compensated for the loss incurred by *sigH* in the mutant strain and thereby ensured the similar survival rates of the wild type and *sigH* mutant.

M. smegmatis, as a saprophyte, encounters several environmental stresses in its ecological niche. We examined the expression of *sigH* paralogs after subjecting *M. smegmatis* cultures to different stress conditions. Heat stress induced the transcript levels of *sigH2* (3.2-fold), *sigH3* (2.9-fold), *sigH4* (14-fold), and *sigH7* (12.6-fold) (Fig. 3). A sudden rise in the

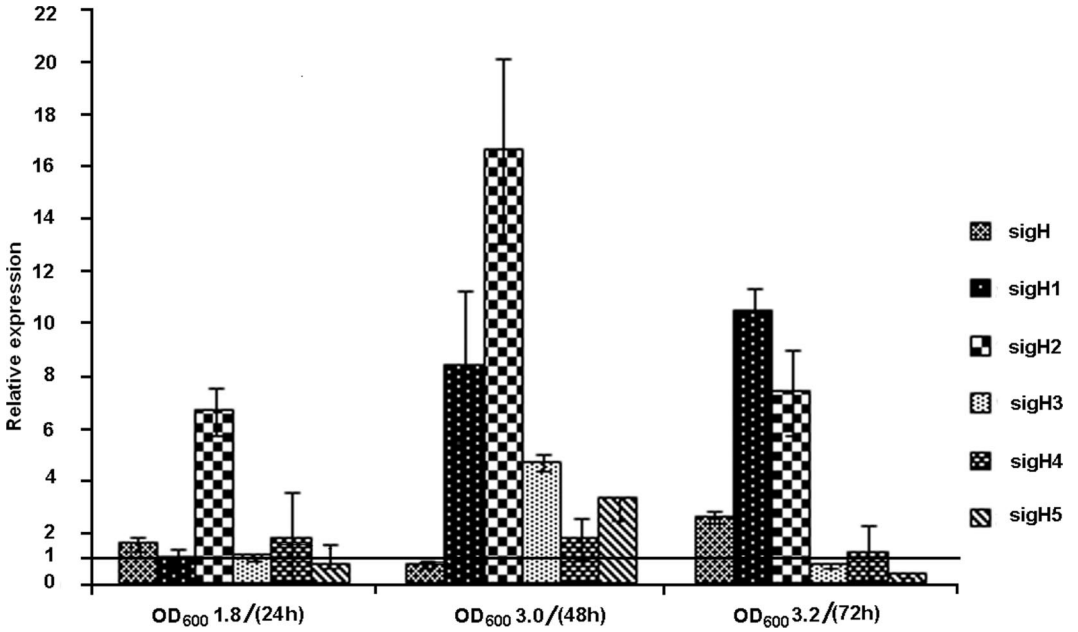

FIG. 2. Relative levels of expression of *sigH* paralogs at the late log (optical density at 600 nm [OD_{600}] of 1.8), stationary (OD_{600} of 3.0), and extended stationary (OD_{600} of 3.2) phases. Expression levels of *sigH* paralogs at early log phase (OD_{600} of 0.5) were considered controls (line at 1 on the *y* axis). *sigH6* and *sigH7* transcripts were far below the control level.

transcription of *sigH4* and *sigH7* upon heat shock suggests a specific role for these sigma factors in regulating the expression of genes that help bacteria overcome heat stress. A low level of *sigH* induction (1.6-fold) during heat stress possibly is the reason behind the similar survival instincts of the *sigH* mutant and its wild-type counterpart after heat shock, as reported earlier (5). Transcript levels of *sigH1* and *sigH5* were relatively comparable to their control levels of expression. Cold shock did not induce the expression of any *sigH* subfamily genes; moreover, *sigH* paralogs except *sigH2* and *sigH3*, whose expression levels did not change, showed a decline in their transcript levels with respect to those of the untreated control. The *sigH6* transcript was at an almost-negligible level under these conditions.

Exponentially growing *M. smegmatis* cultures quickly adapt to a sudden nutrient depletion and can survive for a long period under starvation conditions (21). We simulated nutrient starvation by incubating the *M. smegmatis* cultures in phosphate-buffered saline. This treatment stimulated levels of *sigH*

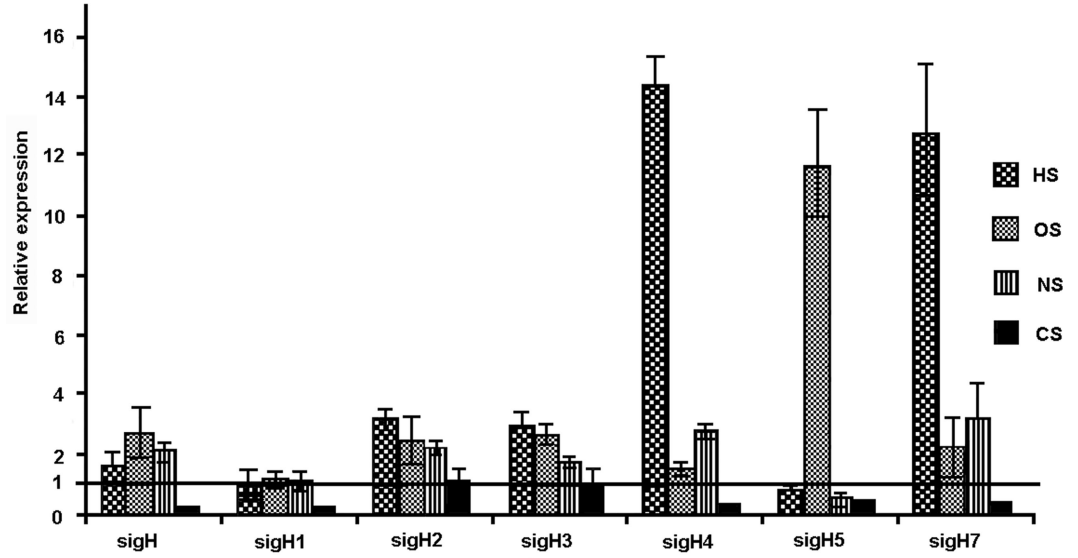

FIG. 3. Relative levels of expression of *sigH* paralogs during heat shock (HS), oxidative stress (OS), cold shock (CS), and nutrient starvation (NS). Note the severalfold increases in *sigH4* and *sigH7* levels during heat shock and in the *sigH5* level during oxidative stress. The *sigH1* level remains unchanged, and other genes are variably induced. Expression levels without treatment were taken as controls (line at 1 on the *y* axis). The *sigH6* transcript remains almost undetectable.

(2.1-fold), *sigH2* (2.2-fold), *sigH3* (1.6-fold), *sigH4* (2.8-fold), and *sigH7* (3.2-fold) expression, while *sigH1* remained unaltered and *sigH5* showed a decline compared to its expression in the untreated control (Fig. 3). The *sigH6* transcript remained almost undetectable.

M. smegmatis mounts a protective oxidative-stress response (19). An *M. smegmatis sigH* mutant showed more susceptibility to organic peroxides than to hydrogen peroxides (5). To examine the role of *sigH* paralogs in hydrogen peroxide-mediated stress, we treated *M. smegmatis* cultures with H_2O_2 and monitored the expression of *sigH* paralogs under treatment conditions. The majority of *sigH* paralogs responded to oxidative stress, a marked feature of *sigH* family proteins. We observed increased levels of expression of *sigH* (2.7-fold), *sigH2* (2.5-fold), *sigH3* (2.6-fold), and *sigH7* (2.2-fold), while *sigH1* levels remained almost unchanged and *sigH4* levels marginally increased with respect to levels in the untreated control (Fig. 3). A dramatic rise in *sigH5* (12-fold) transcript level was particularly noticeable, as its expression did not increase during any other stress conditions applied. It may be recalled that *sigH5* showed enhanced expression during stationary phase and that *M. smegmatis* stationary-phase cells were shown to respond better to hydrogen peroxide stress (21). Interestingly, *M. smegmatis* sigma factors SigB and SigF, which were also shown to render resistance to peroxide stress, showed increased expression during stationary phase (7, 12). These findings reinforce the notion that bacteria adapt to particular stress conditions by regulating the expression of different subsets of genes, orchestrated by an overlapping network of ECF sigma factors. It is tempting to speculate a role for *sigH5* in a transcriptional network that confers protection against hydrogen peroxide stress in *M. smegmatis*.

M. smegmatis is naturally less susceptible to some of the primary-line antimycobacterial drugs, like isoniazid and rifampin (10, 14). An interdependence of oxidative-stress response and isoniazid resistance has been reported to occur among mycobacterial species (3, 4). In *M. tuberculosis*, an extensive transcriptional network responding to oxidative stress was shown to be regulated by SigH (17). To investigate the role of *M. smegmatis sigH* paralogs in response to various drugs, we examined the expression of these sigma factors after subjecting *M. smegmatis* cultures to critical concentrations of antimycobacterial drugs. Antibiotic stress stimulated various transcript levels of *sigH* paralogs, among which an exemplary increase in *sigH1* expression in response to all four of the antibiotics isoniazid (7.5-fold), ethambutol (24-fold), rifampin (30-fold), and streptomycin (17-fold) was particularly noticeable (Fig. 4). Earlier, enhanced *sigH1* expression was noticed only during the stationary and extended stationary phases, not during other stress conditions applied. Since *sigH1* responded markedly to different antibiotics with dissimilar modes of actions, it is difficult to speculate on a role for *sigH1* in mounting a unifying mechanism whereby a single transcription factor might confer resistance to multiple drugs. In *Bacillus subtilis*, another saprophytic soil microbe like *M. smegmatis*, an ECF sigma factor, SigW, showed enhanced expression in response to several antibiotics with different modes of actions and to toxic peptides (9). SigW was found to regulate the expression of an ~60-gene regulon, which encodes proteins that inactivate, sequester, or eliminate toxic compounds from the cell (1). It is possible that *sigH1* controls the expression of genes which provide intrinsic resistance to a range of antimicrobial compounds and other toxic products that cells accumulate during stationary phase and upon exposure to different kinds of drugs. This needs to be further studied. Another noticeable observation was the maximally induced level of *sigH6* (ninefold) in response to isoniazid treatment. It may be recalled that *sigH6* transcripts were found to be at negligible levels during different stages of growth and upon exposure to other stress conditions applied in this study. Since *sigH6* did not respond to ethambutol, it appears that common cell wall biosynthesis inhibitors do not regulate *sigH6* transcription. Notably, isoniazid affects the cell wall biosynthesis process by inhibiting the FAS-II elongation pathway of mycolic acid biosynthesis. It would be of interest to examine the status of *sigH6* transcripts in response to other FAS-II pathway inhibitors. Further studies are required to understand the role of SigH6 in helping *M. smegmatis* overcome isoniazid stress, as it is more tolerant to this antimycobacterial drug.

SigH regulates the transcriptional network that responds to oxidative, nitrosative, and heat stresses in mycobacteria (13, 17). An *M. smegmatis sigH* mutant showed increased sensitivity to oxidative stress by cumene hydroperoxide but no defect in survival at 53°C or oxidative stress due to hydrogen peroxide (5). However, an *M. smegmatis sigH sigE* double mutant was impaired in survival after both heat shock and oxidative stress by cumene hydroperoxide (5). SigH has been shown to be responsible for the stress-inducible expression of SigE, and both SigH and SigE are required for the full induction of SigB (17). Recently, a *sigF* mutant of *M. smegmatis* was found to be significantly impaired in survival after a 50°C heat shock and hydrogen peroxide treatment (7, 15). It is possible that SigF of *M. smegmatis* either belongs to a SigH-mediated network of transcriptional regulation or provides a separate, additional mechanism of response to these stress conditions. The role of SigF and SigH in the resistance of *M. smegmatis* to oxidative stress is also highlighted with the recent observation that the promoter of *dps*, a gene expressed under conditions of oxidative stress and starvation, can be recognized only by RNA polymerase containing one of these sigma factors (2). These findings highlight the regulatory overlap of different sigma factors that work together to allow adaptation to heat and oxidative stress in mycobacteria.

In view of the apparent redundancy of sigma factors mounting overlapping stress responses, we examined the expression of different *sigH* paralogs in *M. smegmatis* and observed a diverse and distinctive expression of *sigH* paralogs during growth and under different stress conditions. The majority of them responded to heat shock and oxidative stresses, a particular feature of the SigH family of sigma factors. Some of them showed a fairly higher-level expression during the stationary and late stationary phases, presumably to impart a better tolerance to these cells against changing physiological states. Overlapping expression of these sigma factors under similar physiological and stress conditions reinstates the earlier reported redundancy of ECF sigma factors. However, an exemplary rise in the expression of *sigH4* and *sigH7* in response to heat shock, *sigH5* in response to H_2O_2, *sigH1* in response to antibiotics, and *sigH6* in response to isoniazid treatment suggests that, probably, these sigma factors are specifically regu-

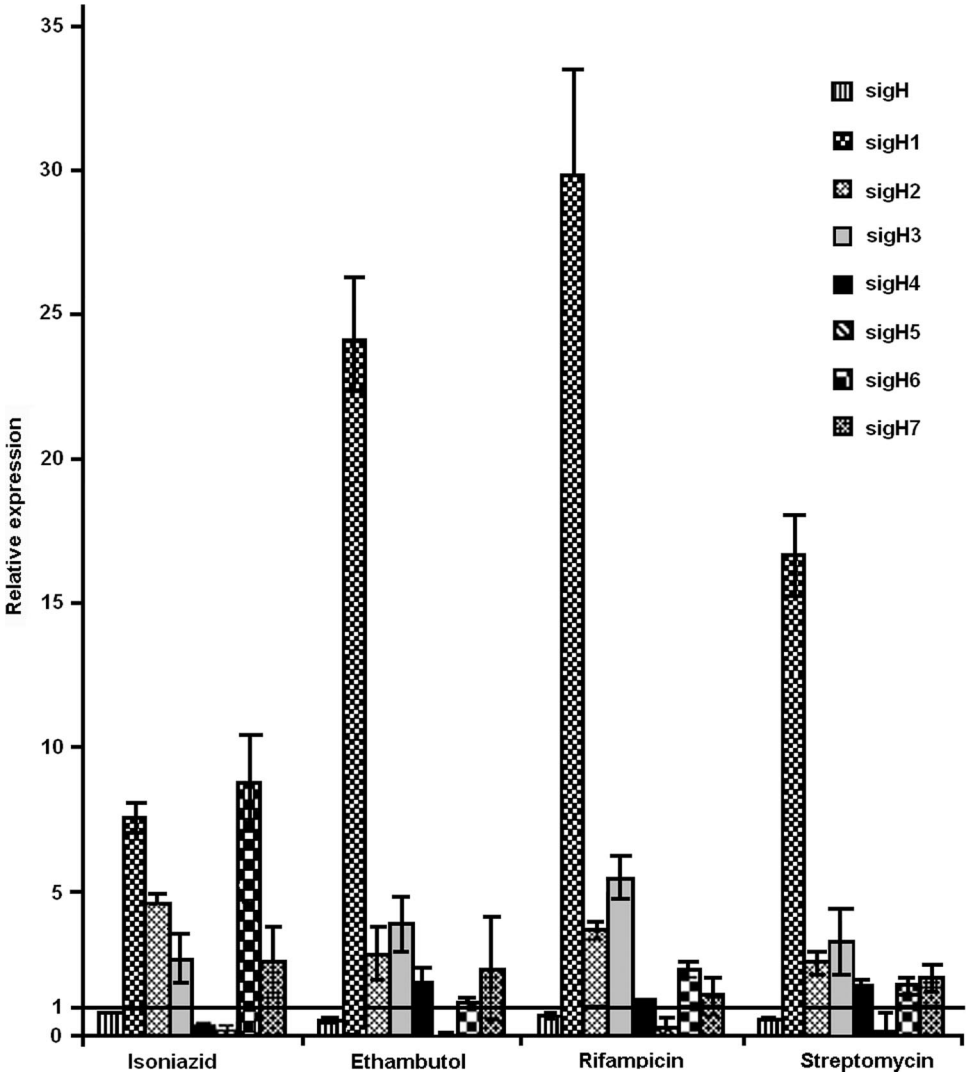

FIG. 4. Levels of expression of *sigH* paralogs upon exposure to antimycobacterial compounds relative to untreated-control expression levels (line at 1 on the *y* axis). *sigH1* is highly induced in response to all antibiotics, while other genes show various levels of expression. The *sigH6* transcript is maximally induced in response to isoniazid.

lated by appropriate signaling molecules to orchestrate the regulation of a selected set of genes. ECF sigma factors are cotranscribed with one or more negative regulators and were reported to interact with the products of their neighboring genes residing in the same operon (24). Often, these include a transmembrane protein with an extracytoplasmic sensory domain and an intracellular inhibitory domain functioning as an anti-sigma factor that binds and inhibits its cognate sigma factor (16). SigH activity is transcriptionally regulated at its autoregulated promoter and posttranslationally via interaction with its cognate anti-sigma factor, RshA, whose gene resides in the same operon (13). The presence of cupin domain protein genes downstream of *sigH1*, *sigH5*, and *sigH7* is noteworthy. Transcriptional regulators with cupin domains were reported to be present in several microbial genomes: *M. tuberculosis* (Rv3833, AraC family of transcriptional regulators), *B. subtilis* (*ydeC*), and *Pseudomonas aeruginosa* (*pae-1*, a heat shock regulator) (11). Interestingly, MSMEG_3484, a cupin domain protein gene present downstream of *sigH1* in its putative operon also possesses an AraC binding domain and is hypothesized to be a transcriptional regulator. Transcriptional regulators of the AraC family were shown to regulate the expression of several stress-responsive genes in bacteria (6). It would be of interest to study the interactions of the cupin domain proteins and other membrane proteins, whose genes are present in the vicinity of *sigH* paralogs, and examine their roles as putative anti-sigma factors to their cognate sigma factors. It would help us decode the signaling cascade operating to orchestrate the complex regulatory circuits of heat and oxidative stress through *sigH* subfamily members. The generation of mutants with single and multiple deletions of *sigH* paralogs with subsequent transcriptome analysis would enable the identification of the regulon of these *sigH* family proteins. A more comprehensive study is required to delineate the overlapping network of a regulon mediated by *sigH* paralogs in *M. smegmatis* during growth and in response to various stress condi-

tions. It would reveal the biological significance of an unusual expansion of the *sigH* family of sigma factors in *M. smegmatis*.

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