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Stress response of genes encoding putative stress signaling molecules of *Mycobacterium tuberculosis*

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1. ABSTRACT

Mycobacterium tuberculosis possesses six genes (*Rv0516c, Rv1364c, Rv1365c, Rv1904, Rv2638 and Rv3687c*) encoding putative anti-sigma factor antagonists or stress signaling molecules (SSMs). We have previously shown that the products of these genes physically interact between themselves and with sigma factor SigF (encoded by *Rv3286c*) and anti-sigma factor RsbW (encoded by *Rv3287c*) in the yeast two-hybrid system. In order to understand whether *ssms* respond to stress, we analyzed the expression of these genes in *M. tuberculosis* exposed to stress at message level using real time RT-PCR. The results revealed that most *ssms* of *M. tuberculosis* responded to stress and *Rv0516c* was the most prominent one. *Rv0516c* showed elevated expression for NaCl, oxidative and starvation stresses and this was followed by *Rv2638* which exhibited upregulation towards stationary phase, heat and oxidative stresses. While *Rv1904* and *Rv3687c* responded significantly to cold and oxidative stresses, *Rv1364c* responded only to heat stress. Further, studies on the response of *sigF* and *rsbW* to stress revealed that only *rsbW* significantly responded to heat, cold, oxidative, starvation and anaerobic stresses. The response of *ssms* and *rsbW* to different stresses may be an indication for the stress activation and regulation of SigF by these molecules.

2. INTRODUCTION

Alternate sigma factors are bacterial transcription factors which help regulate expression of genes in response to a variety of stress conditions (1-3). The activities of these sigma factors are in turn controlled by anti-sigma factors or anti-anti-sigma factors (4,5). Some of these molecules have Ser/Thr kinase and phosphatase activities and they physically interact to transduce the signals similar to eukaryotic signal transduction systems (3). Existence of this signaling system in prokaryotes was first uncovered during the characterization of the general stress sigma factor SigB and the sporulation sigma factor SigF in *Bacillus subtilis* (2,3,6). Termed as 'partner switching' (7), this signaling system involves a network of protein-protein interactions and reversible phosphorylation reactions. Each partner switching module consists of a serine phosphatase, a switch protein/serine kinase and an antogonist protein (2,6). The RsbU-RsbV-RsbW module of *B. subtilis* which communicate environmental stress to SigB is a typical example for partner switching. Recently, partner switching mediated signaling cascades, similar to that of *Bacillus* SigB, have been described from other gram positive bacteria like *Staphylococcus* (8,9), *Listeria* (10) and *Bordetella* (11). Interestingly, partner switching in these organisms is associated with regulation of virulence.

Table 1. Primers used

Gene	Forward 5′→ 3′	Reverse 5′ → 3′
Rv0516c	GACTACGGTGGCGCTCAGAT	GGCCACCTAGCTGTTCGACT
Rv1364c	GTACGGCGTTACGCATGCAG	CGCCGGTTGGTTCGACATAC
Rv1365c	AACGCGCTCAAGGCGACCAT	TCGAGGCCGTTGAGGTTGAC
Rv1904	TGACCTGCATAGCGGCCTAC	GCCACGATCGGCTGGTTACT
Rv2638	ACAGAACCACGCTCTAGTCC	CTCACCAGACGCAGGTCGAT
Rv3286c	GATCGTTCAGCGGTGCTTGC	GATAACCTCAGCGCGGTCCA
Rv3287c	CACCTTCGAGGACCTGGATT	GGTCAGGACATGCCAGCTAA
Rv3687c	CACCTTCGAGGACCTGGATT	GGTCAGGACATGCCAGCTAA
16SrRNA	AAGAAGCACCGGCCAACTAC	TCGCTCCTCAGCGTCAGTTA

M. tuberculosis genes that encode putative partner switching orthologs, identified through Clusters of Orthologous Groups (COG) database, have recently been described (12,13). This group includes the gene *rsbW* (*Rv3287c*), that encodes the antisigma factor RsbW (also known as UsfX) and six genes (*Rv0516c, Rv1364c, Rv1365c, Rv1904, Rv2638 and Rv3687c*) encoding antisigma factor antagonists or stress signaling molecules (SSMs). *rsbW* (*Rv3287c*) is located in an operon with *Rv3286c,* that encodes sigma factor SigF, and RsbW directly interacts with SigF and inhibits SigF dependent transcription *in vitro* (12). Further, RsfA and RsfB, products of *Rv1364c* and *Rv3687c*, respectively, of the *ssm* category, have been shown to interact with RsbW (12). To further delineate the role of SSMs in the regulation of SigF, we studied the interaction of all six SSMs in a yeast two-hybrid system (13). This revealed that most SSMs interact with both SigF and RsbW, suggesting that they transduce signals to these molecules. However, the exact stress signals that they transduce remain unknown. Since most bacterial molecules associated with stress regulation respond to stress, we thought that the response of *ssms* expression to stress would provide some indirect clues. Thus, this study was undertaken to understand the response of *ssms* to different kinds of stress at the transcriptional level. The response of genes encoding RsbW and SigF of *M. tuberculosis* to different stresses was also studied to understand their relationship with *ssms*.

3. MATERIALS AND METHODS

3.1. Mycobacterial culture

Mycocbacterium tuberculosis was grown in Middlebrook 7H9 broth (Difco) containing 0.2% glycerol, oleic acid-albumin dextrose-catalase supplement (OADC) and 0.05% Tween 80 (7H9-OADC-TW) at 37°C in roller bottles.

3.2. Stress experiments

Unless specified otherwise, log phase (0.600 OD at 600 nm) *M. tuberculosis culture* was used to study the effect of different stresses. Before exposing to stress, cultures were harvested by centrifugation and resuspended in fresh 7H9-OADC-TW broth. Tubes, in triplicates, containing 5 ml *M. tuberculosis* cultures were exposed for 1 h to determine the effect of each stress condition. Effects of heat and cold stress were studied by incubating the cultures at 52°C and 4°C, respectively. All other stresses were studied by incubating the cultures at 37°C with appropriate stress components (NaCl, ethanol, hydrogen peroxide (Sigma), cumene hydroperoxide (Sigma), methyl viologen (Sigma), S-nitrosoglutothione (GSNO; Sigma)). Starvation stress was performed by resuspending *M. tuberculosis* in phosphate buffered saline (PBS) and incubating the culture at 37°C for different time periods. Anaerobic stress was studied by incubating the cultures in BBL anaerobic gas pouch for different periods of time.

3.3. RNA extraction

RNA from *M. tuberculosis* was extracted using Tri reagent (Sigma). *M. tuberculosis* control cultures and cultures subjected to stress were pelleted by centrifugation, washed with cold 20 mM Tris-HCl (pH. 8.0) buffer and transferred to RNase free 2 ml screw cap vials with 'O' ring. One ml of Tri reagent and 50 µl of sterile silica particles were added to the pellets, and the cells broken in a bead beater for 2 min. Following this, the tubes were immediately centrifuged at 10,000 rpm and the supernatant containing RNA was extracted with chloroform. The aqueous phase was mixed with 0.6 volume (v/v) of isopropyl alcohol and the RNA pelleted by centrifugation. After mild air drying, RNA pellet was dissolved in RNase free water and the concentration determined at 260 nm in a spectrophotometer. DNA contamination in RNA samples was eliminated by treating the total RNA with DNase I (Invitrogen) prior to cDNA synthesis.

3.4. cDNA synthesis

cDNA was synthesized by reverse transcription using SuperScript™ First-Strand Synthesis System (Invitrogen) and gene-specific antisense primers (Table 1). One µg of DNase I-treated RNA was used in the reaction. Heat inactivated reaction mixture containing cDNA was used to determine the transcript levels in Real-time PCR.

3.5. Real-time RT-PCR

We used an ABI Prism 7900HT sequence detection system (Perkin Elmer) to determine the expression of genes belonging to *ssms* and related genes. We designed primers (Table 1) to amplify approximately 150-250 bp fragments of genes belonging to *ssms*, *rsbW*, *sigF* and *16S rrnA* genes of *M. tuberculosis***.** cDNA was synthesized using gene specific primers (antisense primers of all 9 genes) from total RNA isolated from *M. tuberculosis*, at different growth phases or exposed to different stresses, and this was subjected to real time RT-PCR to determine the transcript level of each gene. Cybergreen reagent (Perkin Elmer) was used for the

detection of transcripts in the ABI sequence detection system. Threshold cycle or C_T in the exponential phase of amplification was used as a criterion to determine the transcript levels and C_T obtained with *16S rrnA* was used to normalize the C_T values for other transcripts. Finally, results were expressed as relative change in expression of genes. Students T test was performed to analyze the significance of differences between data.

4. RESULTS

4.1. Expression of *ssms* during growth of *M. tuberculosis*

In order to understand whether growth phases, particularly the stationary phase, of *M. tuberculosis* have any effect on the expression of *ssm* genes, we first determined the transcript levels of *ssms* from RNA of *M. tuberculosis* at different growth phases. The gene expression profile depicted in Figure 1A shows that *Rv2638*, which was slightly induced towards stationary phase, was the only *ssm* gene that expressed differentially during the growth of *M. tuberculosis*. *sigF* and *rsbW* also did not show any upregulation in their expression even at late stationary phase. Nontheless, *rsbW (Rv3287c)* and *Rv2638* were the only two genes which maintained their expression regardless of growth phases. All other genes seemed to show reduced levels of expression during late stationary phase.

4.2. Expression of *ssms* in response to physical stress

In contrast to growth phases, heat, cold and NaCl stresses exhibited upregulation of certain *ssms* (Figure 1B). While *Rv1364c, Rv2638* and *Rv3287c* showed weak to moderate response to heat stress, *Rv1904, Rv3287c* and *Rv3687c* showed more than four fold increases to cold stress. On the other hand, *Rv0516c* showed over fourteen fold induction in response to NaCl stress, although no other *ssms* showed any response to this stress. Ethanol stress also showed no significant effect on many of the *ssms* tested except a weak induction of *Rv3287c*.

4.3. Expression of *ssms* in response to oxidants

Oxidant stress showed both upregulation and downregulation of *ssm* genes (Figure 1C), although downregulation is not significant. While hydrogen peroxide exhibited a very slight induction of *Rv0516c* and *Rv2638*, cumene hydroperoxide (CHP) showed a differential effect. It slightly downregulated the expression of *Rv0516c, Rv1364c, Rv1365c and Rv2638* and significantly (more than 4 fold) induced the genes *Rv3287c (rsbW)* and *Rv3687c*. The superoxide generator methyl viologen also exhibited a differential effect by slightly inhibiting the expression of *Rv3687c* and inducing the expression of the genes *Rv0516c, Rv1904* and *Rv2638* (showed more than 4 fold induction). However, NO donor GSNO (S-nitrosoglutothione) showed no upregulation of any *ssm* gene, although it had a slight inhibitory effect on the expression of some genes.

4.4. Expression of *ssms* during starvation

Effect of starvation on the expression of *M. tuberculosis ssms* was also examined at different time points (Figure 1D). Most genes showed a decreasing trend of expression after 4 h starvation. However, the expression of *Rv0516c* alone significantly increased after 4 h starvation and this was maintained up to 24 h starvation. Further, expression of *Rv3287c* (*rsbW*) was also found to be induced by more than six fold after 24 h starvation.

4.5. Expression of *ssms* during anaerobic stress

Anaerobic condition, in general, showed only a suppressive effect on *ssm* genes (Figure 1E). However, a two fold induction of the gene *Rv3287c* (*rsbW*) was observed at 4 h and after 24 h exposure to anaerobic condition.

5. DISCUSSION

M. tuberculosis is a significant human pathogen that has the ability to survive in hostile environments like macrophages and granulomas, which have antibacterial defenses and deprived oxygen and nutrients, respectively. This capability of *M. tuberculosis* may partly be due to the regulation of various genes by 12 alternate sigma factors in this species. SigF was the first alternate sigma factor to be identified from *M. tuberculosis* (14) and it shows significant identity with SigB and SigF of *B. subtilis* (15). In addition, *sigF* of *M. tuberculosis*, similar to *sigB* of *B. subtilis*, also has the *rsbW* gene on its upstream region. Recently, it has been reported that *rsbW* and *sigF* together constitute an operon and is driven by a SigF dependent promoter *usfXP1* located upstream of *rsbW* (12). A *sigF* deletion mutant has also been created which showed reduced virulence in animal models (16). However, the fact that *sigF* mutant showed similar sensitivity to that of parental strain towards heat, cold, oxidative and anaerobic stresses (16) and failure of *sigF* to respond to above mentioned stresses other than starvation (17,18) created a concern as to whether SigF plays any role in stress protection.

The results presented here demonstrate that all *ssms* and *rsbW*, whose products interact with SigF, respond to stress. Particularly, *rsbW* is very prominent in responding to oxidative, anaerobic and starvation stress. However, *sigF*, as noticed previously showed no response to any of the stress tested (17). This is somewhat surprising because *rsbW* and *sigF* are cotranscribed together by a single (*usfXP1*, Figure 2) SigF specific promoter (12). Theoretically, *sigF* transcripts, in a given situation, should reflect the levels of *rsbW*. The absence of such a reflection, regardless of stress, may suggest that *sigF* transcripts are purposely kept under low profile and probably under some kind of regulation. But the low message level does not seem to affect the function of SigF, because SigF is still activated by stress which is evident from the upregulation of SigF dependent *rsbW* (12) to different stresses. Further, the induction of *rsbW* corroborates with previously published observations by Michele *et al* (19) from *M. bovis* BCG. These authors translationally fused a *M. tuberculosis* DNA fragment that contained *rsbW,* its upstream *usfXP1*promoter region, and part of *sigF* region with *lacZ* in a plasmid and, after transformation in *M. bovis* BCG, showed induction of LacZ with different stresses. This BCG based response of SigF-LacZ fusion to stress

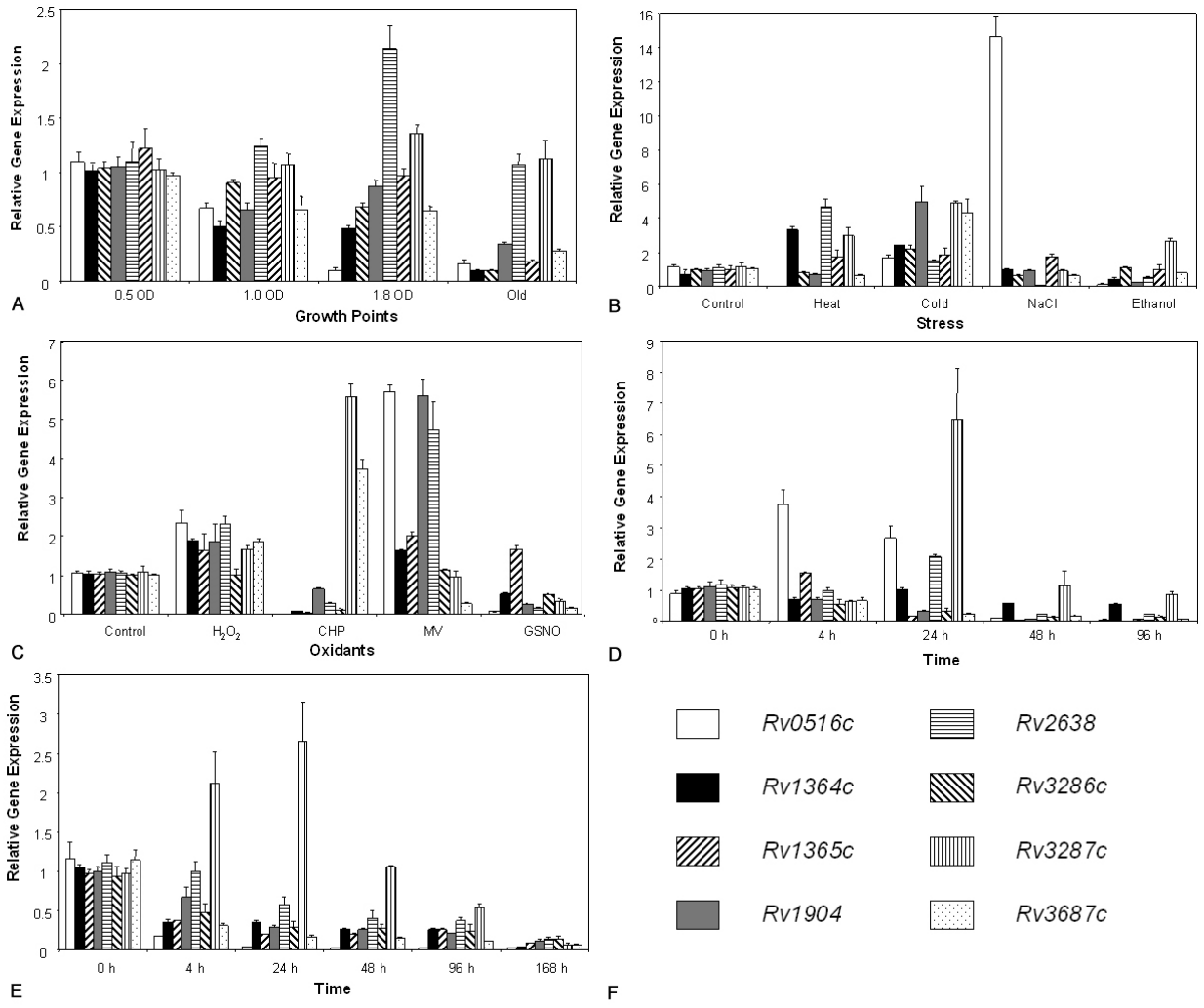

Figure 1. Real-time RT-PCR determination of *ssms*, *rsbW* and *sigF* transcripts from *M. tuberculosis* at different stress conditions. A. During growth of *M. tuberculosis*. 0.5 OD, 1.0 OD, 1.8 OD represent density of *M. tuberculosis* cultures at the time of harvest. 'Old' indicates late stationary phase culture. B. In response to stress. Heat, culture incubated at 52°C for 1 h; Cold, culture incubated at 4°C for 1 h; culture treated with 400 mM NaCl for 1 h; Ethanol, culture treated with 5% (v/v) ethanol for 1h. C. In response to oxidant stress. H_2O_2, culture treated with 5 mM hydrogen peroxide for 1 h; CHP, culture treated with 5 mM cumene hydroperoxide for 1 h; MV, culture treated with 200 mM methyl viologen for 1 h; GSNO, culture treated with 200 mM S-nitrosoglutothione for 1 h. D. During starvation. Starvation experiment was performed by resuspending *M. tuberculosis* in phosphate buffered saline (PBS) and incubating the culture for different time periods. E. Under anaerobic condition. Cultures were incubated inside BBL anaerobic gas pouch for different periods of time. F. Explanation for the bar patterns. The gene representing each pattern is shown under the patterns. Unless specified all experiments were performed with early exponential cultures (0.600OD/600 nm). Each bar represents Mean±SD for three determinations.

may be due to the activation of *usfXP1* promoter by SigF of BCG, since the SigF-LacZ reporter plasmid has only partial sequence for *M. tuberculosis* SigF and can not produce full length SigF needed for the interaction. However, the observed stability of SigF-LacZ in BCG is surprising and it is not clear whether this stability is due to its tarslatioanl nature. Perhaps testing of the SigF-LacZ translational fusion in *M. tuberculosis* to different stresses will clarify this issue better.

The response of *ssms* to different stresses appears complex as most of them respond to multiple stresses (Table 2). *Rv0516c* and *Rv2638* respond to four stresses each, *Rv1904* and *Rv3687c* respond to two stresses each and *Rv1364c* responds to just one stress. It is surprising, however, that *Rv1365c* has shown only a weak response to superoxide (methyl viologen) stress. A previous study has reported that the product of *Rv1365c* (RsfA) binds with SigF under reducing conditions and is predicted to sense redox signals (12). Besides, the response of *Rv1364c* to only heat shock is also unanticipated. Rv1364c is the only SSM which has all four domains required for transmitting signals (13). It has PAS domain in the N-terminal region,

Table 2. Summary of induction of *ssms* in response to stress

Stress	Fold induction							
	Rv0516c	*Rv1364c*	*Rv1365c (rsfA)*	*Rv1904*	*Rv2638*	*Rv3286c (sigF)*	*Rv3287c (rsbW)*	*Rv3687c (rsfB)*
Stationary Phase	-	-	-	-	2.1	-	-	-
Heat	-	3.3	-	-	4.6	-	2.9	-
Cold	-	-	-	4.9	-	-	4.8	4.3
NaCl	14.5	-	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-	2.6	-
Hydrogen peroxide	2.3	-	-	-	2.3	-	-	-
Cumene hydroperoxide	-	-	-	-	-	-	4.5	2.5
Methyl viologen	5.7	-	2.0	5.6	4.7	-	-	-
S-nitrosglutothione	-	-	-	-	-	-	-	-
Starvation	2.6	-	-	-	-	-	6.4	-
Anaerobiosis	-	-	-	-	-	-	2.6	-

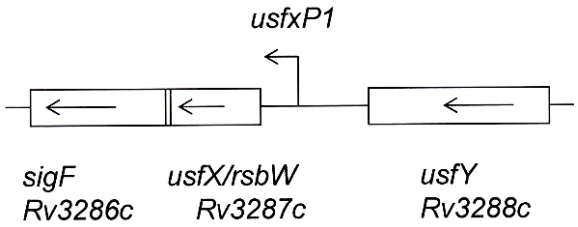

Figure 2. Genetic organization of *sigF* and *rsbW* genes in *M. tuberculosis*. Arrows indicate direction of transcription. SigF-specfic promoter *UsfXP1* (Beaucher *et al*; 2002) is shown on top.

phosphatase and kinase domains in the middle region, and the antagonist domain at the C-terminal end. The whole Rv1364c protein is equivalent to RsbP- RsbW-RsbV proteins of *B. subtilis*. Further, the PAS domain is generally considered as a sensor for redox potential, oxygen tension and energy level (20) and a previous study has reported that *Rv1364c* was upregulated after 96 hours of starvation (18). Moreover, an upregulation of *M. bovis* BCG homologue of *Rv1364c* has been noticed in the macrophage environment (21).

In addition to stress, upregulation of *ssms* were observed when *M. tuberculosis* mutants for regulatory genes were tested for their expression in microarrays. *Rv0516c* was upregulated in *hspR* (22), *sigE* (23) *sigF* (24) and *senX3* (25) deletion mutant strains and *Rv2638* was upregulated in *senX3* (25) deletion mutant strains. Further, an experiment testing the response of *M. tuberculosis* to the antimicrobial agent tetrahydrolipstain in microarrays has noticed down and up regulation of *Rv1365c* and *Rv3687c*, respectively (26). These observations tend to suggest that *ssms* also respond to unconventional stresses and their expression is under multiple regulatory networks.

In summary, our results indicate that most *ssms* respond to stress and it is very likely that their products transduce corresponding signals to SigF. Our results also suggest that SigF is activated under stress conditions. Disruption of *ssms* in the genome of *M. tuberculosis* and stress activation of SigF in *ssm* mutants may provide further insights on the signals that the SSMs transduce to SigF.

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