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Differential Gene Expression in Response to Exposure to Antimycobacterial Agents and Other Stress Conditions among Seven *Mycobacterium tuberculosis whiB*-Like Genes

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The seven *Mycobacterium tuberculosis whiB*-like genes encode small proteins postulated to be transcriptional regulators. A systematic real-time reverse transcription-PCR analysis following exposure to antibiotics and a variety of growth and in vitro stress conditions indicates differential, and in some cases dramatic, transcription modulations for the different *M. tuberculosis whiB* family members. This information together with biochemical analyses of the *whiB1* to *whiB7* gene products will be important for understanding the biology of this novel family of proteins in mycobacteria and related actinomycetes.

Upon infection, *Mycobacterium tuberculosis* has the ability to adapt to many different environments within the host organism. Tubercle bacilli are able to avoid immune system detection and persist inside the host for decades. Many of the conditions to which the bacteria are exposed, such as the acidic environment within the phagolysosomal compartment, are harsh (14). In the lung, there is a recruitment of activated macrophages to the infection site, and these, along with other immune cells, contain the infection by forming a tuberculous granuloma (49). *M. tuberculosis* is adept at surviving within the hypoxic and fatty acid-rich granulomatous environment (8), a facet critical to its ability to persist despite immune pressure (25, 28). Moreover, tubercle bacilli can survive for long periods of exposure to low-nutrient conditions, such as may occur within a granuloma (4, 34), as well as temperature and oxidative stress which may act to disrupt the *M. tuberculosis* cell membrane. The bacterium also has a remarkable tolerance to a wide range of antibiotics (12, 26). To survive, the organism must sense and respond to exogenous stress conditions. Hence, differential expression of transcriptional regulators, which control sets of genes that respond to environmental stimuli, is an important mechanism of stress survival.

A family of genes which may be key transcriptional regulators in *M. tuberculosis* is the *whiB* gene family. The *whiB*-like genes are exclusive to the actinomycetes, such as *Mycobacterium* and *Streptomyces* spp., and are absent from all other organisms studied thus far (9, 44). *whiB* was identified in *Streptomyces coelicolor* as an essential gene for sporulation of aerial hyphae (9), and early sporulation genes were identified by morphological studies of a collection of mutants unable to form normal gray spore pigment (17). Because their aerial mycelium remained white upon prolonged incubation, these mutants were designated *whi* mutants. *S. coelicolor whiB* encodes an 87-amino-acid polypeptide with attributes suggesting that it may be a DNA binding protein (9). *S. coelicolor* WhiB and its related homologues, including *S. coelicolor* WhiD, each contain four cysteine residues, a feature common in metal-coordinating DNA binding proteins (33). Indeed, a recent study has found that Sc WhiD binds a [4Fe-4S] cluster under anaerobic conditions similar to the situation with *Escherichia coli* DNA binding regulators SoxR and Fnr (6, 10, 18). These data suggest that WhiB family members may be involved in DNA binding and in interactions with other proteins leading to transcriptional activation. The *M. tuberculosis* genome contains seven *whiB*-like genes (*whiB1* to *whiB7*) (5, 27, 44). Determining the conditions under which the *whiB* family of genes are induced or repressed is an important step in understanding their possible function and role in the regulation of gene expression in *M. tuberculosis* under different environmental stimuli. We conducted a comprehensive analysis of the expression of the seven *whiB*-like genes in *M. tuberculosis* under various growth conditions and in response to several stresses using quantitative real-time reverse transcription-PCR (RT-PCR). Important stimuli examined were antibiotic exposure, nutrient deprivation, acid, ethanol, detergent, oxidative, heat, and low-iron stresses.

M. tuberculosis strain CDC1551 was grown in roller bottles at 37°C in Middlebrook 7H9 liquid medium supplemented with 10% ADC (albumin-dextrose-salt complex; Sigma), 0.5% glycerol, and 0.05% Tween 80. Cultures were grown to an optical density at 600 nm (OD_{600}) of 0.4, divided into 50-ml aliquots, returned to the incubator for 2 h to equilibrate, and then treated with various stress conditions for 1.5 h. Stress conditions were as follows: 0.05% and 0.1% sodium dodecyl sulfate (SDS) (detergent stress), 5 mM diamide (oxidative stress), 1 mM cumene hydroperoxide (oxidative stress), pH 4.5 (acid stress), phosphate-buffered saline (PBS) (nutrient starvation), 2.5% and 5% ethanol, and 42°C (heat shock). Antibiotic stresses were carried out with the compounds for 3.5 h using high-end critical concentrations as follows: 50 μg/ml cycloserine, 12 μg/ml ethambutol, 0.75 μg/ml isoniazid, 7.5 μg/ml streptomycin, and 50 μg/ml kanamycin. The effect of acid stress was examined by washing the cultures twice with PBS, resuspending of cells in complete 7H9 medium at pH 4.5

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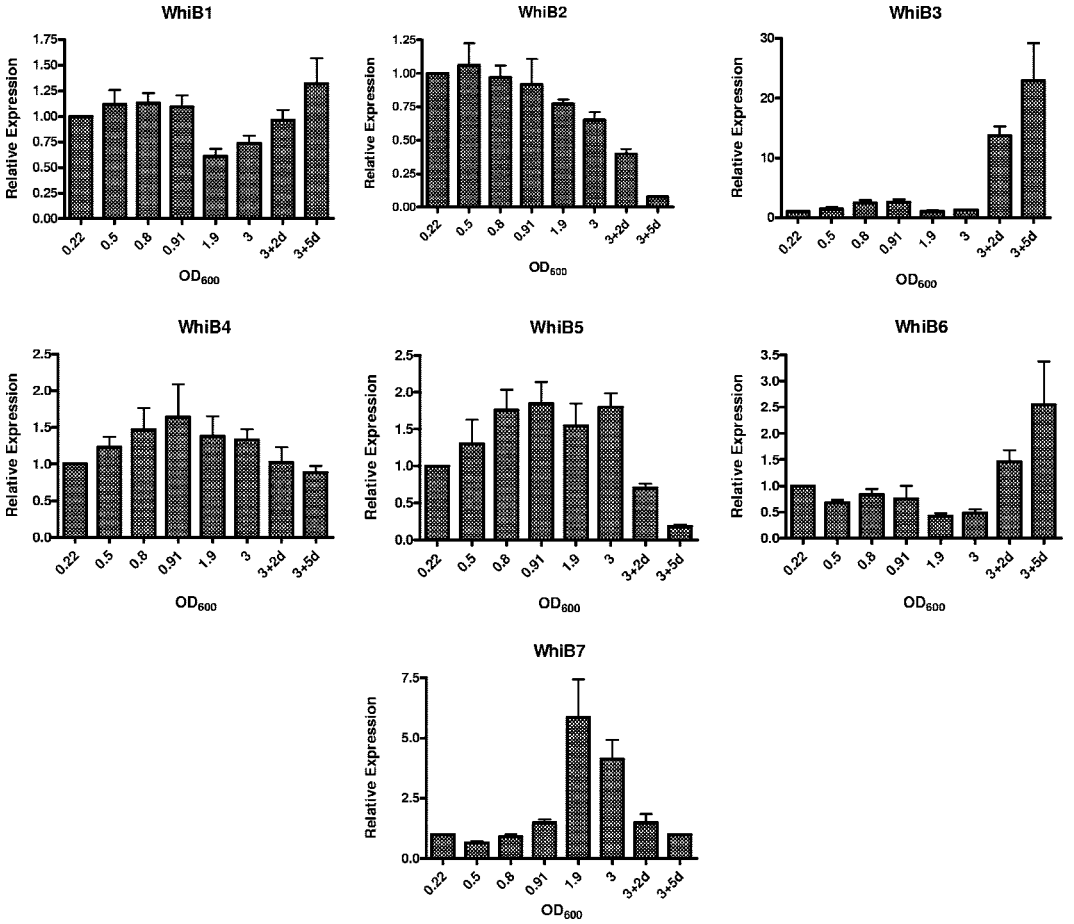

FIG. 1. Relative expression levels of *whiB1* to *whiB7* during various phases of growth. Total RNA was isolated from *M. tuberculosis* (CDC1551) cultures grown to the OD_{600} values shown. The OD_{600} values for the respective growth phases are as follows: early exponential (0.22), mid-late exponential (0.8 to 0.91), early stationary (1.9), late stationary (3.0), and very late stationary (2 days or 5 days after OD_{600} reaches 3.0). Real-time quantitative real-time RT-PCR was performed, and the relative expression level of each *whiB* was calculated using the *sigA* transcript for normalization for RNA amounts and the expression level at an OD_{600} of 0.22 as a control. Data shown are averages from four independent experiments.

(acidified with HCl), and incubating for 1.5 h. For nutrient starvation conditions, bacteria were washed as described above and then resuspended and incubated in PBS for 1.5 h. The cellular response to low-iron stress was studied by washing the cultures twice with PBS, resuspending of cells in GAST (glycerol-alanine-salt) media with and without iron, and incubating for 6 h and 24 h. Cultures were also grown and collected at different growth phases, when the OD_{600} values reached 0.2, 0.5, 0.8, 0.91, 1.9, and 3.0 and 2 and 5 days after the OD_{600} values reached 3.0. RNA was isolated as described previously (47) and treated with DNase I (Invitrogen), and RNA (1 μg) was reverse transcribed using iScript (Bio-Rad). Quantitative real-time RT-PCR was performed with cDNA corresponding to 50 ng RNA using a Bio-Rad iQ SYBR green Supermix kit. Relative expression levels were calculated using the *sigA* transcript for normalization. The average relative expression levels and the standard deviations were determined for four independent experiments.

We first examined the expression of the *M. tuberculosis whiB* family of genes from the early exponential phase to the late stationary phase of growth. Figure 1 shows the relative expression levels of each *whiB*-like gene at the various growth phases. Transcript levels of *whiB1* and *whiB4* remained relatively constant throughout the different phases of growth. *whiB2* expression was constant through early to late-exponential-phase growth, with a gradual decline to almost undetectable levels in late stationary phases. *whiB3* showed a gradual increase of 2.5-fold in late exponential phase and then a decrease to control levels in early stationary phase and a dramatic increase in late stationary phases. *whiB5* levels increased nearly twofold in late exponential and mid-stationary phases, with a decline to very low levels in late stationary phases. There was a decline in *whiB6* expression from control levels to early stationary phase and from this point a gradual increase in late stationary phase to 2.5-fold over control levels. *whiB7* expression increased in late exponential phase to over fivefold during early stationary phase with a decline to control levels in late stationary phases of growth.

Both an abundance of efflux pumps and an unusual cell envelope have been advanced as explanations for the high tolerance of *M. tuberculosis* to antibiotics (1, 19, 31). Recently, however, *M. tuberculosis whiB7* was shown to govern a resis-

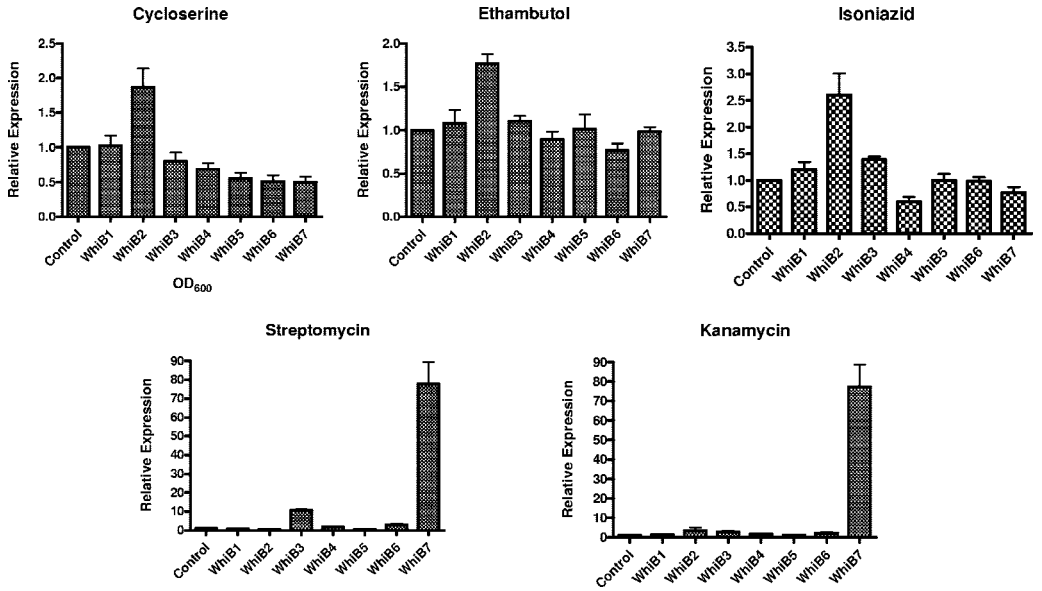

FIG. 2. Relative expression levels of *whiB1* to *whiB7* following antibiotic stresses. Total RNA was isolated from *M. tuberculosis* (CDC1551) cultures (OD_{600}, 0.4) after treatment with ethambutol, isoniazid, cycloserine, streptomycin, and kanamycin for 3.5 h. Real-time quantitative RT-PCR was performed, and the relative expression level of each *whiB* gene was calculated using the *sigA* transcript for normalization for RNA amounts and untreated cultures as a control. Data shown are averages from four independent experiments.

tance mechanism to several well-known antituberculosis drugs (26). To further assess adaptive responses to antibiotics as a mechanism of antibiotic tolerance, we examined the effects of various drugs on the expression of the entire family of *whiB*-like genes. Ethambutol and isoniazid had minimal effects on most of the gene family, but increased transcript accumulation of *whiB2* with both drugs (Fig. 2) and a slight decrease in *whiB4* levels on treatment with isoniazid were observed (Table 1). Cycloserine, another cell wall-acting agent, also stimulated an increase in *whiB2* transcript levels (Fig. 2) and slight decreases in *whiB5*, *whiB6*, and *whiB7* (Table 1). As shown in Fig. 2, streptomycin induced *whiB3* (10-fold), *whiB6* (3-fold), and *whiB7* (70-fold), while *whiB2* and *whiB5* were repressed (Table 1). Kanamycin induced *whiB7* over 70-fold, while *whiB2*, *whiB3*, and *whiB6* increased 2- to 3.5-fold (Fig. 2). Thus, cell wall-active agents (isoniazid, ethambutol, and cycloserine) stimulated *whiB2* transcription, and ribosomally acting aminoglycosides (streptomycin and kanamycin) led to the induction of *whiB7*.

The phagocytosis of *M. tuberculosis* by macrophages in the lung is an early event during infection (16). The phagolysosomes of activated macrophages acidify, and the pH may drop below 6.0 (14, 41, 42), while in certain settings, pathogenic bacteria may interrupt the acidification process (16, 32). Previous transcriptional profiling studies of in vivo-grown *M. tuberculosis* have shown that *whiB4* is upregulated (36). In this study, we examined the effect of acid pH (pH 4.5) on the expression of the *whiB* genes. We found that after 1.5 h of exposure, *whiB3* and *whiB6* were induced 12-fold and 3-fold, respectively, compared to untreated controls. *whiB2* was induced less than twofold. (Fig. 3). The levels for the other *whiB* genes did not vary significantly under these conditions.

M. tuberculosis may be exposed to low-nutrient concentrations within host granulomatous lesions (4, 34). For a model of starvation, we washed *M. tuberculosis* cultures with PBS and incubated them for 1.5 h in PBS (43). Under these culture conditions, we found *whiB2*, *whiB3*, and *whiB5* to be induced about fivefold, twofold, and threefold, respectively, over the untreated controls (Fig. 3). No appreciable variations were

TABLE 1. *M. tuberculosis whiB*-like genes displaying significant (>2-fold) downregulation under growth and stress conditions

Gene	Growth and/or stress condition	Relative expression level
whiB1	0.05% SDS	0.35
	42°C	0.29
whiB2	Late stationary phase (2 days after OD_{600} reached 3.0)	0.40
	Late stationary phase (5 days after OD_{600} reached 3.0)	0.08
	0.05% SDS	0.39
	42°C	0.19
	Streptomycin	0.51
whiB3	Low iron	0.40
whiB4	0.05% SDS	0.27
	5% ethanol	0.47
whiB5	Late stationary phase (5 days after OD_{600} reached 3.0)	0.18
	0.05% SDS	0.31
	2.5% ethanol	0.47
	5 mM diamide	0.41
	Cycloserine	0.55
	Streptomycin	0.44
whiB6	Early stationary phase (OD_{600}, 1.9)	0.42
	Stationary phase (OD_{600}, 3.0)	0.48
	Cycloserine	0.51
whiB7	2.5% ethanol	0.42
	Cycloserine	0.50

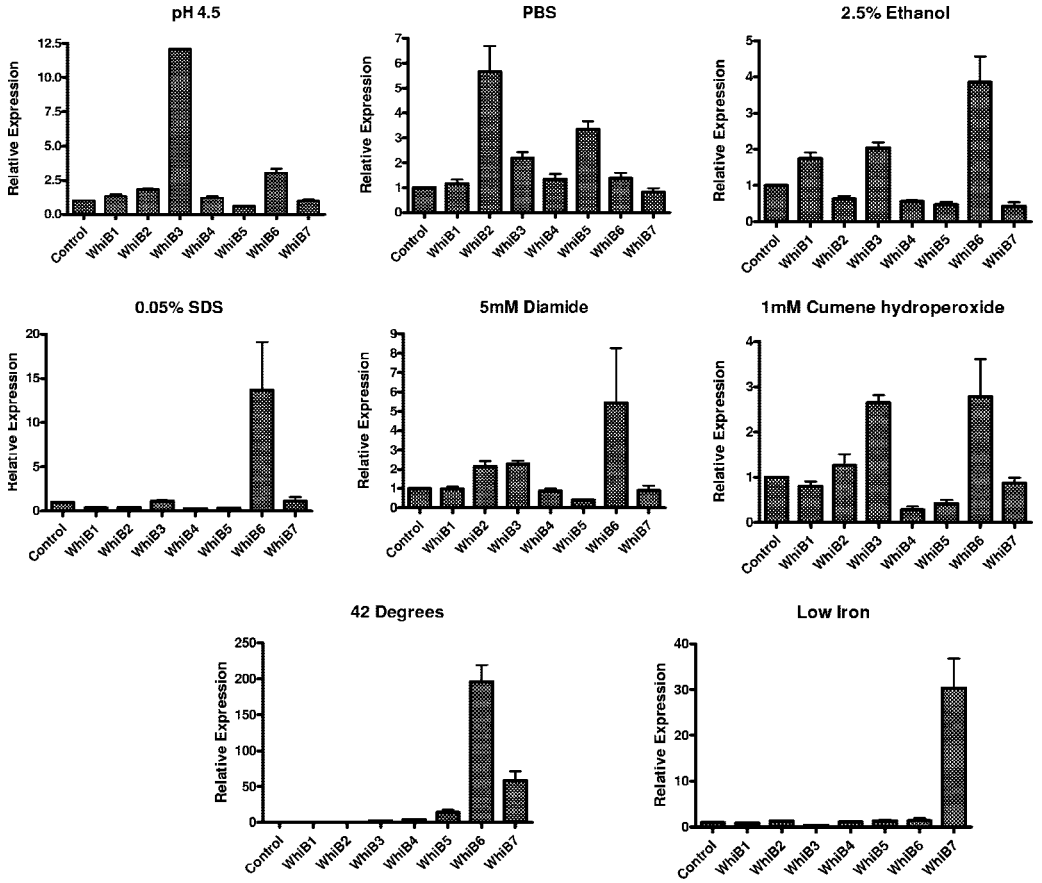

FIG. 3. Relative expression levels of *whiB1* to *whiB7* under stress conditions. Total RNA was isolated from *M. tuberculosis* (CDC1551) cultures grown to an OD_{600} of 0.4 and subjected to various stresses as described in the text for 1.5 h or 6 h for low-iron stress. Real-time RT-PCR was performed, and the relative expression level of each *whiB* was calculated using the *sigA* transcript for normalization for RNA amounts and untreated cultures as a control. Data shown are averages from four independent experiments.

observed in the levels for the other members of this gene family under the conditions tested.

Because ethanol is a protein denaturant and causes membrane disruption (3), we used exposure to ethanol as a stimulus to assess the *M. tuberculosis* responses to protein denaturation and membrane disruption. After 1.5 h of exposure to 2.5% ethanol, *whiB1*, *whiB3*, and *whiB6* were induced two- to fourfold compared to controls (Fig. 3). A similar pattern of induction was seen with 5% ethanol (data not shown). Reductions in the levels for *whiB4*, *whiB5*, and *whiB7* were also observed with 2.5% ethanol treatment (Table 1).

Treatment of *M. tuberculosis* with the detergent SDS has been used to mimic detergent stress, which *M. tuberculosis* may encounter in the form of surfactant early on during the process of infection (20, 23). As shown in Fig. 3, only *whiB6* was significantly induced at exposures of 0.05% SDS; similar data were obtained for 0.1% SDS (data not shown). The other *whiB* genes (except *whiB3* and *whiB7*) were significantly reduced with SDS treatment (Table 1).

In order to survive the burst of reactive oxygen species in activated macrophages, *M. tuberculosis* induces a number of stress response genes to protect itself from the damaging effects of these agents (24, 42, 51). In addition, mycobacteria synthesize compounds like mycothiol, which may offer further protection under such hostile conditions (29, 30, 38). The thiol oxidant diamide has been used extensively to study the effects of changes in the oxidation state of thiols (22). In this study, we examined the effect of diamide treatment on the expression of the *whiB* family of genes. We observed that *whiB2*, *whiB3*, and *whiB6* were induced two- to fivefold with exposure to 5 mM diamide for 1.5 h, whereas *whiB5* levels were observed to drop (Fig. 3 and Table 1). We also examined the effect of another oxidizing agent, cumene hydroperoxide, which mimics the alkyl peroxides produced by metabolism of unsaturated fatty acids and nucleic acids (2). On exposure of cells to 1 mM cumene hydroperoxide for 1.5 h, we observed >2-fold induction in the levels of *whiB3* and *whiB6* (Fig. 3).

The heat shock response is an adaptive pathway involved in the survival of cells exposed to a sudden increase in ambient temperature, and heat shock proteins are induced in both the host and the pathogen during the process of infection (45). We investigated the expression of the *whiB*-like genes after exposure to a temperature of 42°C for 1.5 h. Heat stress led to an increase in the expression levels of *whiB3* (2-fold), *whiB4* (4-fold), *whiB5* (14-fold), *whiB6* (196-fold), and *whiB7* (58-fold). Expression levels of *whiB1* and *whiB2* were observed to drop significantly following heat shock (Table 1).

Iron is an essential nutrient for most pathogens, and iron

limitation at the site of infection is an important mechanism of host defense (11, 40, 50). This phenomenon was simulated in vitro by washing *M. tuberculosis* cultures with PBS and resuspending of cells in GAST media with and without iron. We examined the expression of the *whiB*-like genes after 6 and 24 h of exposure to conditions of low iron. Levels of *whiB7* were induced 30-fold and levels of *whiB3* reduced 2.5-fold at the 6-h time point (Table 1). No effect was observed at 24 h subsequent to iron starvation (data not shown).

The WhiB-like proteins are putative transcription factors involved in the regulation of significant cellular processes, such as cell division, pathogenesis, and response to oxidative stress (13, 15, 21, 28, 37, 46). While they are postulated to serve as DNA binding regulatory proteins based upon the presence of a helix-turn-helix motif (9), and there is evidence that *M. tuberculosis* WhiB3 binds to RNA polymerase sigma factor region 4.2 (46), formal proof that they bind DNA and modulate gene expression has not been established. Recently, Morris et al. demonstrated that exposure of *M. tuberculosis* to the antituberculous drug streptomycin, as well as macrolide and tetracycline antibiotics, led to a significant increase in transcription of *whiB7* (26). Microarray analysis demonstrated that upon subinhibitory exposure to tetracycline, the expression of other genes was temporally dependent on the initial induction of *whiB7*, and hierarchical clustering indicated that 12 other genes were *whiB7* dependent (26). Other *M. tuberculosis whiB* family members have also demonstrated conditional upregulation in response to environmental changes (4, 28, 39). These observations further support the notion that WhiB family member gene products may serve as DNA binding regulators which are responsive to exogenous conditions, including antimicrobial stress.

We further extended this theme by conducting a systematic assessment of the transcription of each of the seven *M. tuberculosis whiB* family members under standard growth conditions as well as exogenous stress conditions. Our results show that each of the *whiB* family members has a unique transcriptional response pattern. Moreover, we confirmed a dramatic induction of *whiB7* with ribosomally active antimicrobial agents. Our study shows that in addition to *whiB7*, *M. tuberculosis whiB2* is also responsive to antimicrobial stress. In contrast to *whiB7* induction by ribosomally acting aminoglycoside agents, the expression of *whiB2* was stimulated by exposure to isoniazid, ethambutol, and cycloserine, agents which inhibit cell wall biosynthesis in mycobacteria. *M. tuberculosis whiB2* was also strongly stimulated by starvation and was downregulated by entry into late stationary phase. Earlier work on *whiB2* focused on its *Mycobacterium smegmatis* orthologue, *whmD*, which was shown to be an essential gene for *M. smegmatis* viability and required for septum formation (15). Since septum formation is linked to new-cell-envelope biosynthesis, these observations associating *whiB2* induction to envelope inhibitors are consistent with reports from other bacterial species (7, 35). The involvement of *whiB2* in septation/cell division is also consistent with our results showing *whiB2* expression to be decreased in late stationary phase, when cell division rates decline.

While *whiB2* was downregulated in late stationary phase, our study revealed that *M. tuberculosis whiB3* expression was dramatically upregulated by entry into late stationary phase. This is of biological relevance since in vitro stationary-phase expression patterns could be relevant to chronic infection in vivo. In addition, *whiB3* transcription was induced more than 10-fold by acid-stress, a condition potentially related to that of in vitro-grown cultures in late stationary phase when culture medium becomes acidified. WhiB3 has been shown to interact with domain 4.2 of the *M. tuberculosis* principal sigma factor and to be required for full virulence in animal models of tuberculosis (46). In the study of *whiB3* genetic deletion by Steyn et al., it is noteworthy that the greatest degree of virulence attenuation was seen in guinea pigs infected with *whiB3* mutants of *Mycobacterium bovis*. Since guinea pigs develop necrotic granulomatous lesions which are rich in fatty acids (48), it is possible that *whiB3* functions as a sensor of acid stress, as may be seen in granulomatous lesions, and that its expression is required for mycobacterial survival in this inhospitable environment.

M. tuberculosis whiB6 was the paralogue which displayed the greatest degree of stress modulation in our study. Like *whiB3*, *whiB6* was strongly induced in late stationary phase, although in contrast to that of *whiB3*, its expression was only modestly increased by acid stress. However, *whiB6* showed strong upregulation in response to SDS stress (~12-fold), heat shock (~200-fold), ethanol exposure (~4-fold), and oxidative stress due to diamide (~5-fold) or cumene hydroperoxide (~3-fold). Neither *M. tuberculosis whiB6* nor orthologous family members related to it in *Streptomyces* have been studied to date. Our study suggests that this particular family member is strongly responsive to a wide variety of stress conditions and may play a key role in generalized stress responses by mycobacteria.

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