The mycobacterial transcriptional regulator whiB7 links redox homeostasis and intrinsic antibiotic resistance*

Ján Burian1,2, Santiago Ramón-García1,2, Gaye Sweet1,2, Anaximandro Gómez-Velasco2,3, Yossef Av-Gay1,2,3 and Charles J. Thompson1,2

1From the Department of Microbiology and Immunology and the 2Centre for Tuberculosis Research, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z3

3From the Department of Medicine, Division of Infectious Diseases, University of British Columbia, Vancouver, British Columbia, Canada

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To whom correspondence should be addressed: Charles J. Thompson. Department of Microbiology and Immunology and the Centre for Tuberculosis Research, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z3, Tel.: (604) 822-2501; Fax: (604) 822-6041; E-mail: charles.thompson@ubc.ca;

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Background: The whiB7 gene is a multidrug resistance determinant in mycobacteria.
Results: WhiB7 autoregulates its own promoter in response to both antibiotics (in a structure and target independent manner) and to redox changes.
Conclusion: WhiB7 links cell metabolism, redox homeostasis, and antibiotic resistance.
Significance: Understanding antibiotic-induced metabolic stress responses in mycobacteria may lead to therapies for mycobacterial diseases including tuberculosis.

SUMMARY

Intrinsic drug resistance in Mycobacterium tuberculosis limits therapeutic options for treating tuberculosis. The mycobacterial transcriptional regulator whiB7 contributes to intrinsic resistance by activating its own expression and many drug resistance genes in response to antibiotics. To investigate whiB7 activation, we constructed a GFP reporter to monitor its expression, and used it to investigate the whiB7 promoter and to screen our custom library of almost 600 bioactive compounds including the majority of clinical antibiotics. Result showed whiB7 was transcribed from a promoter that was conserved across mycobacteria and other actinomycetes including an AT rich sequence that was likely targeted by WhiB7. Expression was induced by compounds having diverse structures and targets, independent of the ability of whiB7 to mediate resistance, and was dependent on media composition. Pretreatment with whiB7 activators resulted in clinically relevant increases in intrinsic drug resistance. Antibiotic-induced transcription was synergistically increased by the reductant dithiothreitol, an effect mirrored by a whiB7-dependent shift to a highly reduced cytoplasm reflected by the reduced:oxidized mycothiol ratio. These data provided evidence that intrinsic resistance resulting from whiB7 activation is linked to fundamental changes in cell metabolism.

The continued rise and prevalence of antibiotic resistance has progressively limited the use of available antibiotics and highlighted a need for new directions in the search for novel and effective antimicrobial therapies (1). Antibiotic resistance is typically mediated by proteins that are transcriptionally activated in response to drug exposure. Chemical inhibitors of drug-activated regulatory proteins or the products of corresponding resistance genes can potentiate antibiotic activity (2,3). Studies of antibiotic activities have traditionally focused on their abilities to inhibit specific targets essential for
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bacterial growth including cell wall biosynthesis, transcription, translation, or DNA replication, with the implicit assumption that these are the direct causes of growth arrest or cell death (4). However, antibiotics exert diverse secondary effects that might provide signals of metabolic distress. In *Escherichia coli*, antibiotics that inhibit ribosomal function induce heat or cold shock responses (5). Importantly, antibiotics alter cell metabolism by activating transcription of up to one in twenty promoters in a concentration- and medium-dependent manner (6). These global changes of gene expression can help predict an antibiotic’s mode of action (7). Furthermore, recent studies indicate that oxidative stress induced by bactericidal antibiotics might be a common cause of cell death (8). Understanding the activation and function of the global response to antibiotic exposure may provide new strategies to overcome antibiotic resistance.

*Mycobacterium tuberculosis*, the etiologic agent of tuberculosis, is intrinsically resistant to most clinically available antibiotics. Intrinsic resistance systems include the low permeability of the cell envelope, drug exporters, drug modifying systems and target modifying enzymes (2). Intrinsic antibiotic resistance to any given drug may be determined by an interactive network including effector proteins, regulatory proteins, and inducers (9). WhiB7, the focus of our study, is a transcriptional regulator of genes that contribute to intrinsic antibiotic resistance in mycobacteria (10).

The WhiB-family of transcriptional regulators is found only in actinomycetes. These include benign and pathogenic mycobacteria as well as *Streptomyces*, producers of the majority of known antibiotics. In mycobacteria, the WhiB-family has vital roles in fundamental cell processes including cell division, redox homeostasis, virulence, and antibiotic resistance (10-12). Little is known about the signals that activate transcription of the whiB genes.

Upon antibiotic treatment, whiB7 has an essential role in activating transcription of antibiotic resistance systems for tetracyclines, macrolides, lincosamides and aminoglycosides (10). Transcription of whiB7 is induced by antibiotic treatment as well as a variety of stress conditions such as heat shock, iron starvation and entry into stationary phase (13). Importantly, whiB7 is highly induced in clinical isolates of the *M. tuberculosis* complex soon after infection of resting and activated murine macrophages (14). The observation that constitutive expression of *whiB7* in trans activated transcription of its native genomic allele suggested autoregulation (10). Like other WhiB proteins, WhiB7 contains 4 cysteine residues that can either bind an iron sulfur cluster or form disulfide bridges within the apo-protein (15). WhiB proteins are redox sensitive and play critical roles in transcriptional activation (12,16,17). Here we identify the essential motifs of whiB7-dependent promoters and explore the mechanism by which the WhiB7 protein responds to diverse antibiotics and physiologies to activate expression of intrinsic drug resistance genes.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and culture conditions**
*Escherichia coli* TOP10 (Invitrogen) was used for cloning. It was cultured in LB broth (Sigma) supplemented with appropriate antibiotics shaking at 200 rpm, 37 °C. Unless otherwise specified, *Mycobacterium smegmatis* mc²155 was cultured in Middlebrook 7H9 broth (Difco) supplemented with 10 % ADC, 0.2 % (v/v) Glycerol, 0.05 % Tyloxapol, and appropriate antibiotics at 37 °C either shaking in flasks at 200 rpm or rolling in test tubes. Kanamycin was used at a final concentration of 30 μg/mL, hygromycin at 50 μg/mL, apramycin at 50 μg/mL and ampicillin at 200 μg/mL.

**Cloning**- All PCR was performed using Dynazyme EXT (NEB F-505S) according to manufacturer instructions. Reactions included 5 % (v/v) dimethyl sulfoxide. Restriction enzymes were from New England Biolabs; digests were performed according to manufacturer instructions. Ligations were performed using T4 DNA ligase (Invitrogen #15224-041) overnight at 16 °C. Strain and plasmid constructions are summarized in Table 1. Oligonucleotides used are listed in Table S1.

**Construction of the Lux reporters**- A DNA fragment containing nucleotides +2 to +674 upstream of the annotated start codon of *whiB7* (Rv3197a) was amplified from *Mycobacterium tuberculosis* H37Rv genomic DNA by PCR using the primers TBpromF and TBpromR. The fragment was digested with *AciI* and EcoRI and cloned into pMV361 digested with the same
enzymes to create pMV361-\text{P}_{B7TB}. The \text{EcoRI} fragment containing the luxABCDE genes was isolated from pAmilux (18) and cloned into \text{EcoRI} digested pMV361 and pMV361-\text{P}_{B7TB} to create the HSP60 promoter driven, constitutively active pLUXon and the antibiotic inducible, \text{whiB7} promoter driven pTB674lux, respectively.

Construction of the GFP reporters- A DNA fragment containing the \text{egfp} gene from pEGFP (Clontech) was amplified by PCR using the primers eGFP\text{F} and eGFP\text{R}. The fragment was cloned, using the polymerase added A overhang, into pGEM-Teasy (Promega) to create pAB2. A DNA fragment containing the first three amino acids of the annotated \text{whiB7} (MSMEG\_1953) and 689 nucleotides upstream region was amplified from the genome of \text{Mycobacterium smegmatis} mc\text{c}155 by PCR using the primers smegpromf and smegpromr. The fragment was cloned into pGEM-Teasy to create pAB6. pAB6 was digested with \text{ClaI} and \text{HindIII} and the \text{whiB7} promoter containing fragment was cloned into pMycVec1 digested with the same enzymes to create pAB7. pAB7 was digested with \text{HindIII} and \text{XbaI} and the \text{egfp} containing DNA fragment was cloned into pAB7 digested with the same enzymes to create antibiotic inducible, \text{whiB7} promoter driven pMS689GFP.

The \text{egfp} from pMS689GFP was amplified by PCR using the primers HSPGFP\text{F} and HSPGFP\text{R}. The PCR fragment was digested with \text{BamHI} and \text{EcoRI} and cloned into pMV261 digested with the same enzymes, to create the HSP60 promoter driven, constitutively active pGFPon.

Sub-cloning the GFP reporter promoter- Shorter fragments of the upstream \text{whiB7} region were amplified from pMS689GFP. The PCR product of GFPsub\text{R} and 438\_F was digested with \text{AclI} and cloned into \text{ClaI} and \text{EcoRV} digested pMycVec1 to create pMS438GFP. The PCR products of 497\_F and 483\_F in combination with GFPsub\text{X}_{\text{R}} were digested with \text{ClaI} and \text{XbaI} and cloned into \text{ClaI}/\text{XbaI} digested pMycVec1 to create pMS497GFP and pMS483GFP, respectively.

Complementation of \text{whiB7} KO strain- The \text{whiB7} gene including its 520 bp upstream and 152 bp downstream regions was amplified by PCR from the \text{M. smegmatis} genome using primers B7comp\_F and B7comp\_R. The PCR fragment was cloned into pMV361, replacing the HSP60 promoter, using \text{XbaI} and \text{HindIII} to construct p361comp. A 1347 bp region of pAB707 containing the apramycin resistance gene \text{aac(3)IV} was amplified by PCR using the primers 797\text{apra}_{\text{F}} and 797\text{apra}_{\text{R}}. The product was cloned into p361comp using \text{HindIII} and \text{SpeI} to construct p361comp\text{apra}. The integrative p361comp\text{apra} was then used to complement the pMS689GFP containing \text{M. smegmatis} \text{whiB7} KO.

Construction of the \text{ermMT} over-expression strain- \text{Rv1988} (\text{ermMT}) was amplified from \text{M. tuberculosis} H37Rv genomic DNA by PCR using the primers Rv1988\_F and Rv1988\_R. The PCR product was digested with \text{EcoRI} and \text{HindIII}, and cloned into pMV361 digested with the same enzymes. The resulting vector was digested with \text{MfeI} and \text{ClaI}, and the \text{ermMT} containing fragment was cloned into pMV361\_hyg digested with the same enzymes to create pERM\_hyg. \text{M. smegmatis} was transformed with the plasmid, transformants were selected for by hygromycin and an increased resistance to macrolides was checked.

Luminescence Timecourse- \text{M. smegmatis} harbouring pTB7lux was inoculated into 3 mL of 7H9 + kanamycin and grown for 55-60 hours, followed by an incubation at room temperature for 12-15 hours. The culture was diluted to an OD600 nm of 0.01-0.005 into 3 mL of kanamycin free 7H9 and grown to an OD600 nm of 0.6-0.8, \text{~20-24} hours. Finally, the culture was diluted to an OD600 nm of 0.2 and 200 \text{µL} was distributed into a black, clear bottom 96 well plate (Costar 3631). The appropriate compound was added to the desired concentration and the plate was placed immediately into a VarioskanFlash (Thermo Scientific). Using the SkanIt RE 2.4.3 software the VarioskanFlash was set to raise the temperature to 37 °C and without waiting shook the plate for 15 seconds at 420 rpm, followed by measuring the OD600 nm and finally measuring luminescence for 1000 ms. The shaking, OD reading and luminescence reading was repeat 12 times at 30 minute intervals, three times at 1 hour intervals, and finally, twice at 3 hour intervals. \text{M. smegmatis} wild type and \text{whiB7} KO harbouring pLUXon were prepared in identical fashion but diluted to an OD600 nm of 0.2 after the room temperature incubation and the timecourse run.
Fluorescence Timecourse- *M. smegmatis* harbouring pMS689GFP was inoculated into 3mL of 7H9 + kanamycin and grown for 48-54 hours until a final OD600 nm of 6-8. The culture was diluted to an OD600 nm of 0.01-0.005 into 3mL of kanamycin free 7H9 and grown to an OD600 nm of 0.6-0.8, ~20-24h. Finally, the culture was diluted to an OD600 nm of 0.2 and 200 μL was distributed into a black, clear bottom 96 well plate. The appropriate compound was added to the desired concentration and the plate was covered with Microseal® ‘B’ Film (BioRad MSB1001), placed immediately into a VarioskanFlash, and fluorescence was monitored. Briefly, the temperature was raised to 37 ºC and without waiting the plate was shaken for 15 seconds at 420 rpm, followed by measuring the OD600 nm and finally measuring fluorescence under default settings by excitation at 488nm and measuring emission at 509nm. The shaking, OD measurement and fluorescence measurement was repeated every 30 minutes 30 more times. *M. smegmatis* wild type and *whiB7* KO harbouring pGFPon was prepared in identical fashion but diluted to an OD600 nm of 0.2 after the 48-54 hour incubation and the timecourse run.

**WhiB7 activation assay-** *M. smegmatis* harbouring pMS689GFP was inoculated into 3mL of 7H9 + kanamycin and grown for 48-54 hours to a final OD600 nm of 6-8. The inoculum was diluted to an OD 600nm of 0.0125 in 50mL of kanamycin free 7H9 and grown for ~20 hours to an OD 600nm of 0.6-0.8. The culture was diluted to an OD600 nm of 0.2 and dispensed into a black, clear bottom 96 well plate. Wells 1-11 of row A received 300 μL and the rest of the plate received 200 μL, except for wells in column 12 of row G and H which received 200 μL of sterile media. For the wells that received 300 μL of culture, 3 μL of stock 5 mM compounds was added and 100 μL was used to serially dilute down from row A to row H creating a concentration range of 50 μM to 0.02 μM in a final volume 200 μL. The plate was covered with a lid, wrapped in tin foil, and incubated at 37 ºC, 200 rpm for 5 hours. After the incubation the plate was placed in a VarioskanFlash, the OD600 nm and emission at 509 nm after 488 nm excitation were measured following a shaking for 15 seconds at 420 rpm. The assay is presented graphically in Figure S4.

The OD and fluorescence values were corrected by subtracting the average of the values measured in wells 12 of row G and H (sterile media). The fluorescent values were then standardized to the OD by dividing the fluorescent value of a well by its corresponding OD value. Finally the fold increase of standardized fluorescence was calculated by dividing values by the average of the untreated wells (wells 12 of row A to F). Because EGFP continued to fluoresce in cultures whose OD<sub>600</sub> had been reduced by antibiotic-induced lysis (data not shown), wells with an OD<sub>600</sub> less than one third of the untreated wells were disregarded. The concentration range for each compound was analysed in three biologically independent runs. A compound was considered a WhiB7 activator if there was at least a ≥4 fold increase in fluorescence versus the untreated control in two out of the three replicates.

**MIC determination-** *M. smegmatis* wild type or *whiB7* KO was inoculated into 3 mL of 7H9 and grown for 48-54 hours to a final OD600 nm of 6-8. The culture was diluted to an OD 600nm of 0.005 and 100 μL was added to 100 μL of antibiotic containing 7H9 at two-fold serial dilutions across a 96 well plate (Costar 3370). The plate was incubated for 48 hours and 30 μL of sterile 10 mg/100mL (w/v) Rezazurin solution was added and the plate was incubated for an additional 24 hours. Wells which remained blue were deemed to contain an inhibitory concentration of antibiotic.

**mRNA isolation and quantification-** *M. smegmatis* was grown to an OD600 nm of 0.6-0.8 and split into 30-50 mL aliquots. The appropriate amount of antibiotic was added to selected aliquots and the cultures were incubated for 1 hour. Four times culture volume of GTC buffer (5 M guanidine thiocyanate , 17 mM sodium lauroyl sarcosinate, 28.5 mM trisodium citrate, 0.5 % (v/v) Tween 80, 0.7 % (v/v) 2-Mercaptoethanol) was added and the samples were incubated for 1 hour at room temperature. Samples were pelleted by centrifugation for 10 minutes, 5000 rpm, 4 ºC and the supernatant discarded. The pellets were suspended in 1mL QIAzol (Qiagen #79306) and transferred to a 2 mL screw cap tube (MBP #3488) containing ~100 μL of 0.1 mm glass beads (BioSpec #11079101). The tubes were beaten three times using a MP FastPrep-24 at 6.0 m/s for 45 seconds with 3-5 minute ice breaks. The
samples were centrifuged for 5 minutes at 16,000 g, 4 °C and the supernatants were transferred to phase gel lock tubes (5 Prime #2302830). The tubes were incubated at room temperature for 5 minutes and 0.2 mL of ice cold chloroform was added. The tube contents were mixed by inversion for 15 seconds, incubated at room temperature for 3 minutes and finally centrifuged for 5 minutes at 16,000 g, 4 °C. The upper fraction was transferred to a 1.5 mL eppendorf containing 550 μL of 30 mM sodium acetate in isopropanol, mixed well, and incubated overnight at -20°C. The samples were centrifuged for 10 minutes at 16,000 g, 4 °C, the supernatant discarded, and the pellets were washed with 1 mL ice cold 75 % (v/v) ethanol. Samples were centrifuged for 5 minutes at 16,000 g, 4 °C and the supernatant discarded. The pellets were dissolved in 90 μL RNase free water by incubation at 65 ºC for 10 minutes. Samples were then treated for 30 minutes at 37 ºC with Turbo DNase (Ambion #AM2239) and finally the RNA was isolated by RNAspin mini columns (GE #25-0500-72) according to manufacturer instructions.

The qScript cDNA synthesis kit (Quanta #95047-100) was used to reverse transcribe a total of 100 ng of RNA per 20 μL reaction [25 ºC – 5 minutes, 42 ºC – 30 minutes, 85 ºC – 5 minutes]. The cDNA samples were diluted 1/10 and 2.5 μL was used per 25 μL quantitave PCR reaction. A mix of PerfeCTa SYBR green supermix (Quanta #95054-050), cDNA and primers (1 μM each) was run on a BioRad Opticon2 [95 ºC – 3 minutes, 95 ºC – 30 seconds, 55 ºC – 30 seconds, read, repeated from step two 34 times]. A standard curve of genomic DNA was used calculate concentrations, and a non-reverse transcribed control to estimate DNA contamination. Primers used for whiB7 were prAB47a and prAB48. Concentrations were standardized to an internal control, mysA, using primers prAB49 and prAB50. Fold increase of whiB7 was calculated against a non-treated sample run in parallel.

Transcription start site determination-Transcriptional start site of the antibiotic induced whiB7 promoter was identified by essentially unmodified Directed Mapping of Transcription Start Site (DMTSS) method developed by Mendoza-Vargas et al. (19). Briefly, RNA was isolated from retapamulin treated M. smegmatis by the method mentioned above. About 1.5 μg total RNA was mixed with 700 pmol of a radom hexamer primer (NNNNNN) and the solution was heated to 70 ºC for 10 minutes followed by incubation on ice for 5 minutes. cDNA was generated using Transcriptor reverse transcriptase (Roche 03 351 317 001) according to manufacturer instruction [25 ºC – 20 minutes, 60 ºC – 40 minutes, 85 ºC – 5 minutes] and isolated by the QIAquick PCR purification kit (Qiagen 28106). A guanine tail was added to the 3’ end of the cDNA library using Terminal transferase (Roche 03 333 566 001) according to manufacturer’s instruction [37 ºC – 30 minutes, 70 ºC – 10 minutes] and the cDNA was once again isolated by the QIAquick PCR purification kit. Linear amplification of the tailed cDNA library was carried out using Dynazyme EXT in combination with the enrich_C primer [94 ºC – 10 minutes, 94 ºC – 1 minute, 50 ºC – 1 minute, 72 ºC – 3 minutes, repeated from step two 34 times, 72°C – 10 minutes] and the cDNA was isolated by the QIAquick PCR purification kit. About 15 ng of the DNA was used per 50 μL PCR reaction using Dynazyme EXT with primers TSS_adaptor and whiB7_TSS58 (94 ºC – 3 minutes, 94 ºC – 30 seconds, 60 ºC – 10 seconds, 72 ºC – 30 seconds, repeated from step two 34 times, 72 ºC – 5.5 minutes). A single PCR product of about 200 bp was observed by agarose gel electrophoresis. This product was cloned, using the polymerase added A overhang, into pGEM-Teasy and transformed into E. coli TOP10. Several white transformants visualized on LB+ampicillin+ 20 µg/mL Xgal+0.1 mM IPTG agar plates were transferred to liquid media, and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen 27106). The plasmids were sent for sequencing at the UBC Vancouver campus NAPS unit (http://naps.msl.ubc.ca/) using the M13R primer.

Analysis of mycothiol content- M. smegmatis wild type or whiB7 KO was grown in 100 mL of NE (glucose 10 g/L, yeast extract 2 g/L, casaminoacids 2 g/L, lab lemco powder 1 g/L) supplemented to 0.05% Tyloxapol at 37 ºC, 200 rpm to an OD600 of 2.0. Cultures were then divided into two 50 mL fractions and one fraction received erythromycin to a final concentration of 256 μg/mL. All fractions were further incubated at 37 ºC for 1 hour. Growth was arrested by the addition of an equal volume of pre-chilled water. The fractions were then divided for centrifugation at
4°C; 10 mL for MSH determination, 30 mL for MSSM determination, and 5 mL for NEM labeling (negative control). Pellets were flash-frozen in liquid nitrogen and stored at -80°C until analysis. HPLC analysis of MSH levels was performed as previously described (20).

Nicotinamide adenine dinucleotide quantification-M. smegmatis wild type or whiB7 KO was grown in 50 mL of NE supplemented to 0.05% Tyloxapol at 37 ºC, 200 rpm to an OD600 of 2.0. Cultures were then divided into two 25 mL fractions and one fraction received erythromycin to a final concentration of 256 µg/mL. All fractions were further incubated at 37 ºC for 1 hour and samples taken. Erythromycin treated samples were left to incubate for an additional 1 hour for the 2 hour time point. Concentrations of reduced (NADH) and oxidized (NAD+) forms of nicotinamide adenine dinucleotide were analyzed as previously described (21). Once samples were extracted the enzymatic reaction was carried out in a black, clear bottom 96 well plate (Costar 3631). Samples and standards were analyzed in triplicate, and only 6 wells were prepared and followed at one time. The Varioskan was set to read the absorbance (570nm) every 20 seconds for 5 minutes. Concentrations were calculated from a standard curve ($R^2 = 0.9996$) of NADH (Sigma N6660-15VL) and standardized to dry weight per litre of culture.

RESULTS

Fluorescent and luminous reporters to study whiB7 promoter activity- Enhanced green fluorescent protein (EGFP) and bacterial luciferase (LuxABCDE) were evaluated as reporters to study the whiB7 promoter and its response to diverse drugs. qRT-PCR confirmed that whiB7 transcription was activated in M. smegmatis by erythromycin [an inducer of whiB7 in M. tuberculosis, (10)], but not isoniazid (Table 2). To monitor the promoter controlling the M. smegmatis whiB7 gene, plasmid pMS689GFP was constructed; it encodes the first 9 base pairs of whiB7 and the 689 nucleotide upstream region (i.e., the entire 513 bp intergenic region and the last 176 bp of the proximal upstream gene, uvrD2) fused to EGFP. An alternative reporter plasmid was also constructed (pTB674lux) containing the upstream intergenic region of the M. tuberculosis whiB7 gene fused to the luxABCDE reporter system. To determine whether these constructs contained the antibiotic inducible whiB7 promoter, erythromycin or isoniazid were added to cultures of M. smegmatis containing pMS689GFP (Figure 1A) or pTB674lux (Figure 1B). Erythromycin, but not isoniazid, induced signal in these cultures. Both whiB7 promoter reporter systems consistently displayed similar induction kinetics, resulting in 4-6 fold increases in signal after 3 hours of induction with erythromycin (Figure 1). Furthermore, the level of erythromycin-induced pMS689GFP fluorescence was much lower in a whiB7 mutant (Figure 1C; whiB7 KO) but was restored upon complementation (Figure 1D). This demonstrated that pMS689GFP could be used to monitor antibiotic-induced transcriptional activation of the whiB7 promoter and provided further evidence that WhiB7 played an essential role in activating its own promoter.

To test whether the EGFP or LuxABCDE reporter activities might be dependent on whiB7, both reporters were expressed constitutively from the M. tuberculosis HSP60 promoter (constructs pGFPon and pLUXon) in M. smegmatis wild type or whiB7 KO strains. As expected, pGFPon generated indistinguishable levels of fluorescence in wild type and whiB7 KO strains (Figure 1E). In contrast, light output by pLUXon was much lower in the whiB7 KO strain background (Figure 1F). This surprising observation revealed that optimal light production by the LuxABCDE protein complex was dependent on physiological conditions determined by whiB7. In addition, a limited screen of our antimicrobial compounds revealed that at least one drug, the IκB-α phosphorylation inhibitor BAY 11-7085, abolished bioluminescence from both pTB674lux and pLUXon in M. smegmatis at concentrations below its minimum inhibitory concentration (MIC) (Figure S1). These results showed that the LuxABCDE reporter system was dependent on whiB7 and that anti-microbial compounds could also have direct or indirect inhibitory effects on its activity. While these observations may lead to future insights into the role of whiB7 or drugs on mycobacterial physiology, it was clear that pTB674lux was not a suitable reporter of whiB7 promoter activity and pMS689GFP was chosen to monitor whiB7 transcriptional activation.

Mapping the antibiotic-responsive whiB7 promoter region- An alignment of the sequences
upstream of whiB7 in the genomes of various Mycobacterium species revealed conserved sequences resembling promoter (-10 and -35) hexamers and an AT-rich region (Figure 2A). This motif was located between nucleotides 438 and 497 upstream of the annotated M. smegmatis whiB7 gene. To determine whether this region contained the inducible whiB7 promoter, the 689 bp upstream sequence used to construct pMS689GFP was shortened to 497 (pMS497GFP), 483 (pMS483GFP), or 438 (pMS438GFP) nucleotides. Only pMS497GFP responded similarly to pMS689GFP when treated with erythromycin (Figure 2B). This was also observed using a broad range of whiB7 activators including retapamulin, tilmicosin, doxycycline, linezolid, A23187 and acivicin (Figure S2). A considerably reduced fluorescence response was generated by pMS483GFP, a construction lacking the conserved AT-rich region (Figure 2B), whereas no fluorescence was detected with pMS438GFP lacking the putative whiB7 promoter (Figure S3).

The transcriptional start site of the antibiotic-induced whiB7 promoter was mapped to a position 445 nucleotides upstream of the gene (Figure 2C). The region contained the predicted whiB7 promoter and this conserved motif was identified upstream of whiB7 in 12 representative actinomycete genera (KEGG database: http://www.genome.jp/kegg; Figure 2D). In all cases, there was an AT-rich region three bases upstream of the conserved -35 site, TTGNNN, and a conserved -10 site, TANNNT. The AT-rich motif was present three bases upstream of the well characterized eis promoter (22), and also upstream of other genes within the whiB7 regulon (tap and ermMT) (10) (Figure 2E).

Activation of the whiB7 promoter is independent of antibiotic structure, primary target of inhibition, and the ability of whiB7 to mediate resistance- To investigate whiB7 transcriptional activation, a custom compound library (Sweet library) was assembled and screened using the pMS689GFP reporter system. The 591-compound library (listed in Table S2) included the majority of commercially available antibiotics targeting DNA, RNA, protein, cell envelope synthesis, or essential metabolic conversions, as well as other physiologically active compounds. Unlike other chemical libraries that are dissolved exclusively in dimethyl sulfoxide, each compound in the Sweet library was dissolved in an optimal solvent (water, dimethyl sulfoxide, ethanol, methanol, or dimethylformamide) to ensure solubility and maximize hit discovery. A semi-high-throughput screen was devised to assay the library for activators of whiB7 transcription. Each compound was serially diluted in 96 well plates in order to survey a broad (2,500 fold) concentration range (50 μM to 0.02 μM; Figure S4). pMS689GFP fluorescence was monitored after 5 hours, when the culture had reached a plateau of signal intensity (Figure 1A). Hits were identified as compounds that induced a ≥ 4 fold increase in fluorescence in at least two out of three trials. In total, 86 whiB7 activators were identified (Table S2). The reliability of the screen was validated by qRT-PCR analysis of 23 representative hits confirming that all induced whiB7 transcription (Figure 3 and Table S2), while 4 non-hits did not (Table S2).

Structure clustering analyses (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?p=clustering) allowed visualization of potential similarities in the chemical structures of whiB7 activators (Figure 3). Compounds with statistically similar structure, suggesting similar bioactivities, are defined by a Tanimoto score greater than 0.7 (23). The cluster analysis identified groups of active compounds having similar structures (including macrolides, aminoglycosides, fluoroquinolones and tetracyclines). However, the structural similarity between these major clades, as well as most of the other activators, was well below 0.7. Therefore, whiB7 transcriptional activation was not due to a common structural motif of the compounds.

The structural diversity of the identified activators was also reflected in the diversity of their documented targets. Half of the identified whiB7 activators, including macrolides, tetracyclines, lincosamides, and aminoglycosides, inhibited protein synthesis, targeting different sites within the 50S or the 30S subunits of the ribosome. The second major target was DNA replication, which was inhibited by fluoroquinolones and DNA intercalators including netropsin, nogalamycin, and pleuromycin. Other potent activators (including acicin, dequalinium, beauvericin, and A23187) are known for their pleiotropic effects on cell metabolism. These data showed that induction of whiB7 transcription did
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not result from direct inhibition of a single target or function, and implied a common, downstream effect of the activators on metabolism. Curiously, cell wall biosynthesis inhibitors, including the extended family of beta-lactams, did not induce transcription.

To provide further evidence for the concept that direct recognition of antibiotic structure was not needed for whiB7 activation, the M. tuberculosis ribosomal methyltransferase (encoded by \textit{ermMT}) that confers macrolide resistance by preventing interaction between macrolide and its ribosomal target (24), was constitutively expressed from the HSP60 promoter in \textit{M. smegmatis} \textit{(ermMT OE)}. If the \textit{whiB7} promoter was induced in response to macrolide structure, independent of translation inhibitory effects, the level of \textit{pMS689GFP} activation would be the same in wild type and in the \textit{ermMT}-expressing, resistant strain. Alternatively, if \textit{whiB7} transcription responded to the stress generated by toxic macrolide-ribosome interactions, macrolides would elicit reduced \textit{whiB7} activation in the resistant strain. When \textit{M. smegmatis} \textit{ermMT OE}/\textit{pMS689GFP} was exposed to various macrolides (erythromycin, dirithromycin, roxithromycin, or azithromycin) at concentrations that activated \textit{pMS689GFP} in the wild type background, the level of induced fluorescence was ~5 fold lower (Figure 4). The level of activation by doxycycline, an antibiotic whose activity is unaffected by ErmMT, was unchanged (Figure 4).

Interestingly, several activators were not within the \textit{whiB7} sensitivity spectrum. For example, while dequalinium, linezolid, danofloxacin, and A23187 were all active inducers of \textit{whiB7} expression, the \textit{whiB7} KO mutant was not more sensitive to these compounds (Table 2). Furthermore, pre-treatment with \textit{whiB7} inducers increased intrinsic resistance levels. Pre-treatment with sub-inhibitory concentrations of erythromycin or clarithromycin increased resistance to those antibiotics in a \textit{whiB7}-dependent manner (Table S3). Similarly, treatment with acivicin, an amino acid analog, also increased resistance to macrolides (4-fold; Table S4).

The fact that the glutamine analog acivicin was a strong inducer of \textit{whiB7} transcription (Table 2), suggested a link between amino acid metabolism and \textit{whiB7} activation. When glutamate, the only amino acid in standard 7H9 medium, was either retained or replaced with glutamine, aspartate, asparagine, arginine, or histidine, and cultures were treated with erythromycin, levels of \textit{whiB7} induction varied; induced levels were similar in cultures grown in the presence of glutamate, aspartate or histidine, whereas increased levels of induction were observed in cultures grown in the presence of asparagine with a limited increase with glutamine and a possible small increase with arginine (Figure S6).

\textbf{Redox state modulates the level of \textit{whiB7} transcriptional activation-} Based on \textit{in vitro} studies showing that WhiB proteins, including WhiB7, have oxygen sensitive iron sulphur clusters (12,15) that may affect transcription (17), and our studies establishing that \textit{whiB7} is needed for regulation of its own promoter, experiments were carried out to explore the hypothesis that oxidizing or reducing reagents (diamide or dithiothreitol (DTT)) might affect \textit{whiB7} promoter activity \textit{in vivo}. In the absence of erythromycin, the thiol reductant DTT moderately induced expression (Figure 5A). Treatment with DTT in combination with a low dose of erythromycin generated a strong, synergistic (~1,300 fold) increase in mRNA levels (Figure 5A). Conversely, the thiol oxidant diamide generated a negligible increase in \textit{whiB7} mRNA and decreased the level of induction by erythromycin approximately 5 fold (Figure 5A). These data indicate that a reduced state favors \textit{whiB7} transcriptional activation.

Kohanski \textit{et al.} (8) reported that hydroxyl radical formation is a common mechanism of antibiotic-mediated bacterial cell death. Because our experiments demonstrated that reducing, rather than oxidizing, conditions enhanced \textit{whiB7} activation, it seemed unlikely that \textit{whiB7} transcription was responding to hydroxyl radicals generated by antibiotic action. In fact, adding the hydroxyl scavenger thiourea at concentrations similar to those used by Kohanski \textit{et al.} to prevent significant hydroxyl radical accumulation, did not prevent \textit{whiB7} transcriptional activation by several antibiotics (Figure S5). Instead, thiourea activated the \textit{pMS689GFP} reporter (Figure S5). This provided further evidence that \textit{whiB7} activation is intimately linked with cell metabolism and activated by a reducing environment.

Mycobiol, the major low molecular weight reducing agent in actinomycetes, provides resistance to free radicals as well as antibiotics.
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(25,26). In mycobacteria, the ratio between its reduced form (MSH) and its oxidized disulfide form (MSSM) serves as an indicator of intracellular redox potential, with a basal reducing ratio of about 200:1 (MSH:MSSM) in M. smegmatis (20). Without an antibiotic inducer, the whiB7 KO contained ~10 fold less MSH and ~2 fold less MSSM (Figure 5B) than the wild type, reflecting in a ~5 fold decrease in MSH:MSSM (Figure 5C). After treatment with erythromycin, MSH levels were unchanged in wild type cells or the whiB7 KO background (Figure 5B). Importantly, erythromycin treatment virtually eliminated detectable MSSM in wild type cells and had no effect on levels in the whiB7 KO (Figure 5B). This resulted in a ~180 fold MSH:MSSM increase in wild type cells (Figure 5C).

To determine whether the reduced MSH:MSSM ratio in the whiB7 KO or the increased MSH:MSSM ratio generated by treatment with erythromycin in the wild type reflected broader effects on redox metabolism, the levels of reduced (NADH) and oxidized (NAD+) nicotinamide adenine dinucleotide were quantified. During exponential growth the whiB7 KO strains contained slightly lower levels (40% decreased) of NADH compared to wild type (Figure S7A). Upon erythromycin treatment, the levels of both NADH and NAD+ increased in both strains. The increase was more pronounced in the whiB7 KO; after 2 hours of erythromycin treatment NADH levels increased ~3 fold and NAD+ levels ~2 fold, while the increases in wild type were ~1.6 and ~1.2 fold, respectively (Figure S7A). The NADH:NAD+ ratio was ~1.6 fold lower in the whiB7 KO relative to wild type in exponentially growing cultures, and the ratio in both strains increased after erythromycin treatment (~1.3 and ~1.7 fold after two hours in the wild type and whiB7 KO; Figure S7B).

**DISCUSSION**

To better understand factors that affect the intrinsic resistance of mycobacteria to antibiotics, we identified essential components of the whiB7 promoter and the spectrum of inducers that activate its transcription using an EGFP reporter system. Our chemical screens and investigations of redox metabolism provided new insights into the biological activation of whiB7 expression. We suggest that inducers generate a reductive shift of redox metabolism in response to inhibition of diverse cellular targets and that this leads to the transcriptional activation of whiB7-dependent intrinsic drug resistance.

Actinomycete genomes all encode WhiB7 orthologs as well as a conserved promoter consensus sequence (Figure 2D), suggesting that whiB7 has an indispensible role in natural environments and that its transcriptional activation system is conserved in this diverse group of soil and pathogenic bacteria. A comparison of the whiB7 promoter to consensus sequences for all M. tuberculosis sigma factors (27) showed that its -35 and -10 hexamer sequences and their spacing was most similar to SigA, the primary vegetative sigma factor. By analogy to WhiB3, WhiB7 may partner with SigA to activate transcription of specialized regulons (28). Since loss of the conserved AT rich region upstream of the -35 hexamer restricted activation (Figure 2B) and WhiB7 contains an AT-hook (10), a motif known to bind the minor groove of AT-rich DNA (29), our results imply that the sequence serves as a WhiB7 binding site. The recognition motif is also found upstream of the genes within the proposed regulon (Figure 2E). The M. smegmatis genome contains 5,954 regions with blocks of at least five adjacent A or T nucleotides (searched with ‘[AT]{5}’ in DNA Pattern Search available at www.geneinfinity.org).

Since many WhiB proteins do not encode an obvious AT hook motif, other conserved regions of WhiB7 may be involved in binding DNA directly or by partnering with other transcriptional activators to target promoters within its regulon. Although antibiotic induction of the whiB7 promoter was strongly reduced in the whiB7 KO background, a slow, constant increase in activity was observed (Figure 1E). This may reflect the participation of other regulatory elements that either repress under non-inducing conditions or co-activate upon induction.

The whiB7 promoter was induced by a wide variety of compounds (Figure 3) at concentrations much lower than their MICs (Table 2). Many whiB7 transcriptional activators perturb respiration, redox balance, or transmembrane ion flux. One of the most intriguing inducers was the glutamine analog acivicin. Acivicin inhibits glutamine amidotransferases leading to a response described as “metabolic mayhem” in E. coli (30). In our M.
smegmatis cultures, acivicin did not inhibit growth at the highest concentration tested (800 μM). The fact that it substantially increased whiB7 transcription even at 16-fold lower concentrations (50 μM; Table S2) clearly illustrated the concept that activation was due to a drug-induced metabolic signal. The observation that not all toxic compounds induced whiB7, throughout a range of growth inhibitory and noninhibitory concentrations, demonstrated that the signal was not a common feature associated with cell death. Active compounds apparently induce a unique whiB7-specific metabolic stress signal. In fact, whiB7 inducers were enriched for compounds that inhibit protein biosynthesis, demonstrated by a dose-dependent effect on ribosomes by macrolides (Figure 4; Figure 5A). Insights into specificity were also provided by the observation that none of the 50+ cell wall targeting compounds in the Sweet library induced whiB7 expression (Table S2). Protein synthesis and cell wall inhibitors are known to induce distinctive stress signals associated with different stress regulons (31,32).

A previous study had demonstrated that treatment of M. smegmatis with sub-inhibitory concentrations of macrolides greatly increased resistance to these drugs in an erm(38) (the M. smegmatis ermMT ortholog; MSMEG_1646) -dependent manner (33). Our work showed that macrolide pre-treatment increased resistance to the activating macrolide in a whiB7-dependent manner (Table S3). The whiB7 promoter motif was present upstream of erm(38) (Figure 2E) implying that the increase in resistance was mediated by erm(38) which requires whiB7 for transcriptional activation. Therefore, a whiB7 response to any activator compound or metabolic signal should similarly increase macrolide resistance. In support of this concept, our data demonstrated that pre-treatment with acivicin, a non-macrolide whiB7 activator, resulted in increased resistance to macrolides (roxithromycin and clarithromycin; Table S4). The clinical relevance of this induced intrinsic resistance phenomenon has recently been demonstrated in multi-drug resistant strains of M. tuberculosis. Pre-treatment with rifampicin induced expression of multiple efflux pumps leading to increased resistance to ofloxacin, a quinolone (34). Rifampicin pre-treatment of these strains increased expression of whiB7, and presumably a multidrug transporter that it activates (tap, Rv1258c) contributing to the resistance state (10,34). These observations support a clinically important concept; exposure to whiB7 inducers activates expression of genes and a metabolic state that provides cross-resistance to diverse drugs.

Since oxidative stress generated by hydroxyl radicals has been reported to be a common metabolic effect of cidal antibiotics in some bacteria (8), we monitored whiB7 expression under reducing and oxidative culture conditions. Interestingly, DTT (providing additional reducing potential) activated a low level of whiB7 transcription (Figure 5A). Combining reducing conditions with a low concentration of erythromycin synergistically increased whiB7 transcription, mimicking treatment by a much higher concentration of erythromycin. In contrast, oxidizing conditions (diamide) decreased induction (Figure 5A). In addition, the hydroxyl radical scavenger thiourea did not prevent antibiotic-induced whiB7 induction (Figure S5). This implied that the whiB7 response to macrolides (and other drugs) was dependent on a metabolic shift to increased reducing potential that was generated independently of hydroxyl radical formation. The reducing potential of mycobacterial cells is reflected in the redox state of reducing agents including MSH and NADH.

Analyses of MSH, MSSH (Figure 5B), and their ratio (MSH:MSSM) (Figure 5C) revealed differences between wild type and whiB7 KO strain, both untreated and in response to erythromycin. These studies revealed that whiB7 participates in maintaining a reduced cytoplasmic (MSH:MSSM) environment under normal growth conditions and directly or indirectly controls the concentration of mycothiol (MSH+MSSM; Figure 5B). Furthermore, the fact that erythromycin treatment of the wild type strain resulted in a whiB7-dependent decrease in the pool of oxidized mycothiol suggested that WhiB7 had an active role in regulating MSSM reduction (Figure 5B). The decrease in MSSM shifted the MSH:MSSM ratio to a highly reduced state (Figure 5C). By analogy to other WhiB proteins, the activity of WhiB7 as a transcriptional activator may respond to such changes in the redox environment. In support of this model, DTT studies indicated that a highly reduced environment potentiated induction of whiB7 transcription (Figure 5A). In contrast, erythromycin treatment of the whiB7 KO did not
affect levels of oxidized mycothiol (Figure 5B) or its MSH:MSSM ratio (Figure 5C). By comparison, changes in NADH and NAD+ were relatively small, suggesting that the whiB7-dependent mycothiol redox changes were an initial response to erythromycin, reflected by slower changes in NADH/NAD+ pools. While increased NADH and NAD+ pools might be needed to provide longer term responses to erythromycin, they were metabolically isolated from MSH/MSSM pools under the condition of our experiment.

It has been reported that catabolism of host fatty acids in macrophages results in reductive stress by the accumulation of NADH/NADPH (12,35). The stress is dissipated by a WhiB3-dependent shift to the production of virulence lipids (12), but the initial reductive burst may explain the activation of whiB7 in the M. tuberculosis complex soon after infection of macrophages (14) or in response to lipids added to the growth media (10). Additionally, macrophages also utilize glutathione as the major antioxidant for defence against M. tuberculosis infection (36). Exposure to glutathione may provide additional reductive stress to facilitate activation of whiB7. Finally, the observation that the whiB7 KO strain has a more oxidized cytoplasm (Figure 5C) and lower levels of NADH (Figure S6A) rationalizes the reduced output of LuxABCDE bioluminescence (Figure 1D) which requires reducing power to generate reduced riboflavin mononucleotide, an essential cofactor for the light generating reaction (37).

Other transcriptional regulators containing redox sensitive disulfide bridges or iron-sulfur clusters serve to regulate critical redox responsive functions in bacteria (38). WhiB1, WhiB2, WhiB3, and WhiBTM4 (a bacteriophage encoded WhiB2-ortholog) proteins also interact with target promoters in a redox-sensitive manner (12,16,17). WhiB7, like all WhiB family proteins, contains 4 conserved cysteine residues that are able to bind a redox sensitive iron-sulfur cluster or form disulfide bonds. By analogy to biochemical studies of WhiB1, the iron-sulfur cluster-bound form of WhiB7 may be an inactive form of the protein that does not bind DNA (17). While our evidence supports the central role of reduced thiols, it does not necessarily rule out the involvement of oxidants. If oxidative damage is a common mechanism of bactericidal antibiotic mediated cell death (8), the cell may respond by shifting its redox balance back to a more reduced state. This might allow activation of WhiB7 to induce expression of other genes in its regulon and thereby generate a defensive response after exposure to some antibiotics.

Studies of antibiotic resistance in bacterial pathogens have traditionally focussed on genes carried by transmissible elements that provide drug resistance to pathogens. However, there is growing recognition that chromosomal genes having physiological functions can have alternative roles as antibiotic resistance genes. This new concept has important evolutionary implications, rationalizing the recruitment and evolution of metabolic genes to serve as antibiotic resistance genes or alternatively, to provide physiological functions for genes under selective pressure as resistance genes. Here we focused on whiB7, a transcriptional regulatory gene that determines intrinsic drug resistance in mycobacteria. Our studies revealed that WhiB7 is at a cross road between physiology and antibiotic resistance with functions linked to the maintenance of balanced reducing potential as well as activation of resistance genes. Activation of whiB7 creates a non-specific resistance state that provides cross-resistance to diverse drugs. Understanding how WhiB7 senses antibiotic-induced signals to activate intrinsic resistance genes may allow more effective uses of drug combinations (39) for treatment of mycobacterial diseases including tuberculosis.

REFERENCES

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Acknowledgements-J.B. designed, performed and analyzed experiments. S.R.G. designed performed the mycothiol and macrolide preincubation studies, and analyzed data. G.S. assembled the Sweet library and analyzed data. A.G.V. performed mycothiol studies, Y.A. designed and analyzed mycothiol data and interpreted results. C.J.T designed experiments, analyzed data and supervised the work. J.B., S.R.G., G.S., C.J.T. wrote the manuscript. We thank Andrea Basler and Jeffery Hu for technical assistance, Dr. Julian Davies for providing pAmilux and Dr. William Jacobs for providing pMV361.hyg. We thank Dr. Steven Hallam for the use of the Varioskan and qRT-PCR machine and Keith Mewis for technical support with the Varioskan. We also thank Carol Ng, Leah Lim and Quinn Parker for critical reading of the manuscript. This work was supported by Canadian Institute of Health Research grant MOP-82855 held by CJT.

FIGURE LEGENDS

FIGURE 1. Analyses of EGFP and LuxABCDE as reporters of whiB7 promoter activity. *M. smegmatis* wild type containing A, pMS689GFP or B, pTB674lux treated with 1 μM erythromycin (Ery), 50 μM isoniazid (Iso) or untreated (Untreated). The erythromycin induced fluorescence of pMS689GFP was decreased in the whiB7 KO (C), a defect that was restored by providing whiB7 in trans (D, whiB7 KO Comp). Signal output from cultures containing E, pGFPon or F, pLUXon in *M. smegmatis* either untreated or treated with 1 μM erythromycin (+ery). Values plotted are the mean ± SEM of triplicate samples. Lines illustrate the trend. Results are representative of multiple transformants.

FIGURE 2. Sites and sequences defining the antibiotic-inducible whiB7 promoter. A, Alignment of a 60 base pair region 438 to 497 nucleotides upstream of *M. smegmatis* whiB7 with sequences from 8 different *Mycobacterium* species. Conserved nucleotides are designated by the star (*) and the mapped transcriptional start site is highlighted by the black box. Here and in the following sections the -10 and -35 regions, and the putative WhiB7 binding site are highlighted in grey. B, Fluorescence response of EGFP under the control of shortened whiB7-promoter regions, pMS947GFP and pMS483GFP, in *M. smegmatis* either untreated or treated with 1 μM erythromycin (+ery). Values plotted are the mean ± SEM of triplicate samples. Lines illustrate the trend. Results are representative of multiple transformants. C, The transcription start site was mapped using RT PCR (19) and the transcript cloned. The sequences of two independent clones (bottom) were aligned to the *M. smegmatis* genome sequence (top). The matching sequence is boxed, the transcriptional start site is marked TSS. D, An alignment of the identified whiB7 promoter motif across 13 genera of actinomycetes. The variable distance from the start codon is boxed E, The putative WhiB7 binding site and promoter motif is present upstream of several *M. tuberculosis* H37Rv genes within the WhiB7 regulon as well as *erm(38)* the ortholog of *ermMT* in *M. smegmatis*.
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FIGURE 3. Structural comparison of identified whiB7 promoter activators. Identified activators were clustered according to chemical structure similarity using PubChem (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?p=clustering). A Tanimoto score of ≥0.7 is indicative of highly similar structure with potentially similar bioactivity. Numerous activators clustered within distinctive structural classes are highlighted (blue, macrolides; red, aminoglycosides; yellow, fluoroquinolones; green, tetracyclines). Activators confirmed by qRT-PCR are indicated by a red star (*).

FIGURE 4. The whiB7 promoter responds to the toxic macrolide-ribosome interaction. M. smegmatis wild type and ermMT OE carrying pMS689GFP were treated with 1.85 µM erythromycin (Ery), 0.62 µM dirithromycin (Diri), 0.62 µM roxithromycin (Roxi), 0.62 µM azithromycin (Azi), or 0.069 µM doxycycline (Doxy) for 5 hours and the fluorescence compared to untreated cultures. All drugs were pretested to define their most effective concentrations for whiB7 induction in M. smegmatis wild type. Values plotted are the mean ± SEM of three biologically independent experiments.

FIGURE 5. whiB7 promoter activation is altered by intracellular redox. A, Increase of whiB7 mRNA in cultures treated with 8 µM erythromycin (Ery8), 1 µM erythromycin (Ery1), 1 µM erythromycin under reducing conditions (10 mM DTT + Ery1), 1 µM erythromycin under oxidizing conditions (Diamide (5 mM) + Ery1), reducing conditions (DTT (10 mM)) or oxidizing conditions (Diamide (5 mM)) relative to a non-treated control. Results plotted are the mean ± SEM of duplicate measurements and representative of three experiments. B, The reduced form (MSH) and the oxidized disulfide form (MSSM) of mycothiol were quantified in M. smegmatis wild type or whiB7 KO. Cultures were either untreated (Untreated) or treated for 1 hour with 256 µg/mL erythromycin (Erythromycin). Results plotted are the mean ± SEM of three biologically independent experiments. C, The redox ratio (MSH:MSSM) was calculated from mycothiol quantities (plotted in ‘B’) of untreated (Untreated) or 256 µg/mL erythromycin treated (Erythromycin) M. smegmatis wild type or whiB7 KO strains. Values are the mean ± SEM.

TABLE 1. Strains and plasmids used

<table>
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<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Mycobacterium smegmatis mc²155</td>
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<td>Unmodified lab strain</td>
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<tr>
<td>whiB7 KO</td>
<td>Genomic region 2031710 to 2032094 (containing MSMEG_1953 (whiB7)) replaced by Hygromycin resistance; Hyg&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(CJT, SRG unpublished)&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>ermMT OE</td>
<td>pERM.hyg integrated into genome constitutively expressing ermMT to provide macrolide resistance; Hyg&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This Study</td>
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<tr>
<td>whiB7 KO Comp</td>
<td>p361comp.apra integrated into the genome of the whiB7 KO strain providing whiB7 expressed under its own promoter in trans; Hyg&lt;sup&gt;R&lt;/sup&gt; Apra&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This Study</td>
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<td>Plasmids</td>
<td>pLUXon</td>
<td>Constitutively expressed luxABCDE using HSP60 promoter in the integrative vector pMV361; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pTB674lux</td>
<td>luxABCDE controlled by the M. tuberculosis whiB7 promoter region (up to 674 nucleotides upstream of Rv3197a) replacing the HSP60 promoter in the integrative vector pMV361; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This Study</td>
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<td>pGFPon</td>
<td>Constitutively expressed EGFP using HSP60 promoter on the multi-copy vector pMV261; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pMS689GFP</td>
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<td>p361comp.apra</td>
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<td>pAB707</td>
<td>Source of the apramycin resistance gene <em>aac(3)IV</em></td>
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*Construction and further complementation of the *whiB7* KO is to be published elsewhere*  

Hyg^R = hygromycin resistance Kan^R = hygromycin resistance Apra^R = apramycin resistance
**TABLE 2.** Comparison of *whiB7* transcriptional activators to the *whiB7* resistance spectrum.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fold increase in susceptibility</th>
<th>Fold increase&lt;sup&gt;b&lt;/sup&gt; of <em>whiB7</em> mRNA (treatment concentration in μM)&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>wild type</td>
<td><em>whiB7</em> KO</td>
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</tr>
<tr>
<td>erythromycin stearate</td>
<td>32</td>
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<td>16</td>
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<td>retapamulin</td>
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<td>6.25</td>
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<td>tilmicosin</td>
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<td>acivicin</td>
<td>&gt;800</td>
<td>&gt;800</td>
<td>-</td>
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<tr>
<td>A23187</td>
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<td>&gt;25</td>
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<td>erythromycin</td>
<td>16-32</td>
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<tr>
<td>isoniazid</td>
<td>116-232</td>
<td>nd</td>
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<sup>a</sup>MICs were determined in three biologically independent samples.

<sup>b</sup>Fold increase in mRNA (ratio of treated/untreated) was determined from a sample analyzed in duplicate after 3 hours of induction (concentration shown in brackets).

<sup>c</sup>Concentrations correspond to the highest fold increase of pMS689GFP fluorescence in the chemical screen.

nd = not determined
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Figure 2

A

M. smegmatis
Mycobacterium mcs
Mycobacterium kms
Mycobacterium fts
M. tuberculosis
M. bovis
M. marinum
M. ulcerans
M. avium_paratuberculosis

M. tuberculosis
Rv3197a
Streptomyces coelicolor
Actinosynnema mirum
Amycolatopsis medierranei
Geodermatophilus obscurus
Gordonia bronchiadis
Salinispora tropica
Micromonospora aurantica
Kyotococcus sedentarius
Tsukamurella paurometabola
Saccharomonospora viridis
Nocardiopsis farcinica
Rhodococcus sp. RHA1 RHA1_r06383

M. smegmatis
MSMEG_1953

B

Fluorescence/OD

Time (min)

C

WblB7 Binding Site

D

M. tuberculosis
Rv3197a
Streptomyces coelicolor
Actinosynnema mirum
Amycolatopsis medierranei
Geodermatophilus obscurus
Gordonia bronchiadis
Salinispora tropica
Micromonospora aurantica
Kyotococcus sedentarius
Tsukamurella paurometabola
Saccharomonospora viridis
Nocardiopsis farcinica
Rhodococcus sp. RHA1 RHA1_r06383

E

Rv2416c - elr
Rv1258c - tap
Rv1988 - ermM7
MSMEG_1646 - erm(38)

WhiB7 Binding Site

Downloaded from http://www.jbc.org/ by guest on August 17, 2017
Figure 3
Figure 4

![Graph showing the fold increase of fluorescence for different macrolides under wild type and ermMTOE conditions.](image-url)
Figure 5

A

Fold increase whiB7 mRNA

B

nmoles/10^6 cells

C

Redox Ratio

(MSH, MSSM)

Wild Type

whiB7 KO

Wild Type

whiB7 KO

Untreated

Erythromycin

Untreated

Erythromycin
SUPPLEMENTAL FIGURES AND LEGENDS

FIGURE S1. LuxABCDE activity is abolished by Bay 11-7085 in M. smegmatis.
Bioluminescence of A, pTB674lux and B, pLUXon in M. smegmatis treated with 0, 2.2
and 11 μM Bay 11-7085. The values plotted are the mean ± SEM of triplicate samples.
Lines are drawn to illustrate trends.
FIGURE S2. Fluorescence of pMS497GFP is induced by many whiB7 activators. Fluorescence response of *M. smegmatis* pMS497GFP either untreated or treated with 25 μM retapamulin (Reta), 1.5 μM tilmicosin (Til), 0.25 μM doxycycline (Doxy), 1.5 μM linezolid (Line), 5 μM A23187 (A23187), or 75 μM acivicin (Aci). Values plotted are the mean ± SEM of triplicate samples. Lines illustrate the trend. Results are representative of multiple transformants.
FIGURE S3. The pMS438GFP reporter lacking the whiB7 promoter does not generate fluorescence. Fluorescence of *M. smegmatis/*pMS438GFP either untreated or treated with 1 μM erythromycin (+ery). Values plotted are the mean ± SEM of triplicate samples. Lines illustrate the trend. Results are representative of multiple transformants.
FIGURE S4. A flow chart of the GFP based *whiB7* promoter activity assay. The pMS689GFP reporter strain was inoculated into 3 mL of kanamycin-containing media and grown to stationary phase. The culture was diluted into fresh antibiotic-free media and grown to early exponential phase. The OD<sub>600</sub> was standardized to 0.2 and the culture dispensed into a clear bottom, 96 well plate that prevented light transmission between wells (black). Compounds (5 mM) were transferred from the Sweet library to the black plate and serially diluted. The black plate was incubated for 5 hours and fluorescence was then measured. Fold increase in fluorescence was calculated and analyzed for activator compounds. Because EGFP continued to fluoresce in cells lysed by antibiotic treatment, wells with an OD<sub>600</sub> less than one third of the untreated wells were disregarded (set as ‘0’). A sample plate is shown with retapamulin and rosamicin identified as activators.
FIGURE S5. Fluorescence response of pMS689GFP to varying concentrations of the hydroxyl radical scavenger thiourea and antibiotic treatment. A, 7H9 containing 0, 50 and 150 mM thiourea. The induced fluorescence observed in response to 150 mM thiourea may be to its reductive effect or to osmotic stress, B, 0.1 μg/mL tetracycline in 7H9 containing 0, 50 and 150 mM thiourea. C, 5 μM A23187 in 7H9 containing 0, 50 and 150 mM thiourea. Values are the mean ± SEM of triplicate samples.
FIGURE S6. Strength of \( \text{whiB7} \) promoter activation is altered by amino acid metabolism. \( M. \text{smegmatis} \) pMS689GFP cultured in 7H9 (- glutamate) supplemented with either aspartate, asparagine, arginine, glutamine, glutamate or histidine were induced with 1 \( \mu \text{M erythromycin} \). Cultures grew at similar rates in all of these media with no amino acid-dependent effects on fluorescence (pMS689GFP) under non-inducing conditions. The fluorescence signal standardized to \( \text{OD}_{600} \) is plotted as the mean ± SEM with the trend highlighted by coloured lines.
FIGURE S7. NADH and NAD+ levels respond to erythromycin in M. smegmatis wild type and whiB7 KO strains. A, The reduced form (NADH) and the oxidized form (NAD+) of nicotinamide adenine dinucleotide were quantified in M. smegmatis wild type or whiB7 KO. Cultures were either untreated (Untreated) or treated for 1 or 2 hours with 256 µg/mL erythromycin (Erythromycin 1h and Erythromycin 2 h). Results are plotted as the mean ± SEM of three biologically independent experiments. B, The redox ratio (NADH/NAD+) was calculated from mycothiol levels (plotted in ‘A’) of untreated (Untreated) or 256 µg/mL erythromycin treated (Erythromycin 1h, Erythromycin 2 h) M. smegmatis wild type or whiB7 KO strains. Values are the mean ± SEM.
### TABLE S1. Oligonucleotides used in the study

<table>
<thead>
<tr>
<th>Oligonucleotide (Forward)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBpromF</td>
<td>CGTAAACGTTCGTTGGTGAGACGTGTGCAGC</td>
</tr>
<tr>
<td>TBpromR</td>
<td>CGTTGAATTCTCCTGTTTCACCTGCTTCTGGTC</td>
</tr>
<tr>
<td>eGFP_F</td>
<td>AAGCTTTATGGTGAGCAAGGGCGAGG</td>
</tr>
<tr>
<td>eGFP_R</td>
<td>TCTAGAGATATCTTACTTGTAAGCTCAGTCATGG</td>
</tr>
<tr>
<td>smegpromF</td>
<td>CTATCGATAAAGTGCCCTGCTTACCTGTTG</td>
</tr>
<tr>
<td>smegpromR</td>
<td>ACAAGCTTAGCAAGTCTCCGCAATGGA</td>
</tr>
<tr>
<td>HSPGFP_F</td>
<td>TATAATGGATCCCATGTGGAGCAAGGGCGAGGA</td>
</tr>
<tr>
<td>HSPGFP_R</td>
<td>TAATAGAATTTCCATATGTCCGCCCTCGGACGGTC</td>
</tr>
<tr>
<td>Rv1988_F</td>
<td>TAATAGAATTTCCATATGTCCGCCCTCGGACGGTC</td>
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<tr>
<td>Rv1988_R</td>
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</tr>
<tr>
<td>GFPsub_R</td>
<td>TAATAGAATTTCCATATGTCCGCCCTCGGACGGTC</td>
</tr>
<tr>
<td>438_F</td>
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<td>GFPsubX_R</td>
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<td>TSS_adaptor</td>
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<td>whiB7_TSS58</td>
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<td>M13R</td>
<td>CAGGAAACAGCATGATG</td>
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*Insights into whiB7 activation and action*
Insights into whiB7 activation and action

**TABLE S3.** Pre-treatment with sub-inhibitory concentrations of macrolides boosted resistance in a whiB7 dependent manner

<table>
<thead>
<tr>
<th>aStrain</th>
<th>bPre-incubation</th>
<th>cMIC (μg/mL)</th>
<th>dFold</th>
<th>dFold</th>
</tr>
</thead>
<tbody>
<tr>
<td>155</td>
<td>None</td>
<td>64-128</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>155-B7</td>
<td>None</td>
<td>4</td>
<td>16-S32</td>
<td>0.125</td>
</tr>
<tr>
<td>155</td>
<td>1/4xMIC155</td>
<td>256</td>
<td>2-4R</td>
<td>32-64</td>
</tr>
<tr>
<td>155</td>
<td>1/4xMIC155-B7</td>
<td>256</td>
<td>2-4R</td>
<td>32-64</td>
</tr>
<tr>
<td>155-B7</td>
<td>1/4xMIC155-B7</td>
<td>4</td>
<td>1</td>
<td>0.125</td>
</tr>
</tbody>
</table>

aM. smegmatis mc²155 wild type (155) or whiB7 KO (155-B7)
bCells were incubated overnight (16-18 hours) in the presence or absence of the indicated amount of macrolide. Cells were assayed against the same antibiotic in which they were pre-incubated. 1/4xMIC155: ERY, 8 μg/mL; CLA, 1 μg/mL. 1/4xMIC155-B7: ERY, 0.5 μg/mL; CLA, 0.01 μg/mL. Medium used was NE (glucose 10g/L, yeast extract 2g/L, casaminoacids 2g/L, LAB LEMCO powder 1g/L).
cMICs were assayed over a range of two-fold serially diluted drugs.
dFold increase in sensitivity compared to the respective non-treated strain for each wild type or whiB7 mutant background. The 155-B7 non-treated was compared against 155 non-treated strain. S, increase in sensitivity; R, reduction in sensitivity.
ERY, erythromycin. CLA, clarithromycin.
TABLE S4. Pre-treatment with sub-inhibitory concentration of acivicin boosted resistance of *M. smegmatis* to macrolides

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>aMIC (μg/mL)</th>
<th>bPre-treated</th>
<th>cFold</th>
</tr>
</thead>
<tbody>
<tr>
<td>clarithromycin</td>
<td>2</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>roxithromycin</td>
<td>8</td>
<td>32</td>
<td>4</td>
</tr>
</tbody>
</table>

aMICs were assayed over a range of two-fold serially diluted drugs.
bEarly exponential cells (~0.3 OD₆₀₀) were incubated in the presence of 100μM acivicin for 6 hours. Cultures were then diluted to 0.005 OD₆₀₀ and MIC determinations carried out against the listed antibiotics. Medium used was 7H9.
cFold increase in resistance as compared to the respective MIC of non-treated *M. smegmatis mc²155* cells
The mycobacterial transcriptional regulator whiB7 links redox homeostasis and intrinsic antibiotic resistance
Jan Burian, Santiago Ramon-Garcia, Gaye Sweet, Anaximandro Gomez-Velasco, Yossef Av-Gay and Charles J. Thompson

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