WhiB7, an Fe-S-dependent Transcription Factor That Activates Species-specific Repertoires of Drug Resistance Determinants in Actinobacteria*

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WhiB-like (Wbl) proteins are well known for their diverse roles in actinobacterial morphogenesis, cell division, virulence, primary and secondary metabolism, and intrinsic antibiotic resistance. Gene disruption experiments showed that three different Actinobacteria (Mycobacterium smegmatis, Streptomyces lividans, and Rhodococcus jostii) each exhibited a different WhiB7-dependent resistance profile. Heterologous expression of whiB7 genes showed these resistance profiles reflected the host’s repertoire of endogenous whiB7-dependent genes. Transcriptional activation of two resistance genes in the whiB7 regulon, tap (a multidrug transporter) and erm(37) (a ribosomal methyltransferase), required interaction of WhiB7 with their promoters. Furthermore, heterologous expression of tap genes isolated from Mycobacterium species demonstrated that divergencies in drug specificity of homologous structural proteins contribute to the variation of WhiB7-dependent drug resistance. WhiB7 has a specific tryptophan/glycine-rich region and four conserved cysteine residues; it also has a peptide sequence (AT-hook) at its C terminus that binds AT-rich DNA sequence motifs upstream of the promoters it targets. Targeted mutagenesis showed that these motifs were required to provide antibiotic resistance in vivo. Anaerobically purified WhiB7 from S. lividans was dimeric and contained 2.1 ± 0.3 and 2.2 ± 0.3 mol of iron and sulfur, respectively, per protomer (consistent with the presence of a 2Fe-2S cluster). However, the properties of the dimer’s absorption spectrum were most consistent with the presence of an oxygen-labile 4Fe-4S cluster, suggesting 50% occupancy. These data provide the first insights into WhiB7 iron-sulfur clusters as they exist in vivo, a major unresolved issue in studies of Wbl proteins.

WhiB-like proteins (Wbl) are found as multiple paralogs in Actinobacteria (1), where they play diverse roles in essential functions, as well as antibiotic resistance (2). The best known Actinobacteria include Streptomyces, which produce the majority of known antibiotics (3), and Mycobacterium tuberculosis, a leading cause of human mortality due to bacterial infection. Streptomyces sp. display diverse specific drug resistance patterns (4), which may be related to the evolution of thousands of antibiotic biosynthetic pathways in their genomes (3). Other saprophytic Actinobacteria such as Mycobacterium smegmatis (5) and Rhodococcus jostii may require intrinsic drug resistance determinants to survive in various soil ecosystems that may contain antibiotics and toxins produced by competing organisms.

Chemotherapeutic options for tuberculosis are limited by the efficient intrinsic antibiotic resistance system of M. tuberculosis (6, 7). Many of these resistance phenotypes require WhiB7 (7) and are linked to redox metabolism (8). Disruption of whiB7 sensitizes M. tuberculosis to several antibiotics with different chemical structures and mechanisms of action, whereas overexpression promotes resistance (7). More extensive screens using M. smegmatis as a model system identified other whiB7 activators, including compounds that perturb respiration, redox balance, transmembrane ion flux, as well as heat shock and iron starvation (9, 10). Antibiotic exposure caused autoinduction of the whiB7 promoter and a whiB7-dependent increase of cellular thiol reducing power (9). Some of these inducers may alter cellular redox conditions comparable with those encountered by M. tuberculosis during host macrophage

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invasion or chemotherapeutic treatments. Indeed, whiB7 is highly up-regulated in macrophages (11) and in the lungs of infected animals (12). WhiB7 activates expression of its own regulon that includes resistance genes involved in antibiotic efflux (tap, Rv1258c) (13), ribosome modification (erm, Rv1988) (14, 15), modulation of the host immune responses (eis, Rv2416c, and erm) (16, 17), and mycobacterial survival within macrophages (eis) (7).

In addition to drug resistance, Wbl genes have roles in morphogenesis, cell division, virulence, and both primary and secondary metabolism. Two members of the family, whiB and whiD, were first identified in Streptomyces coelicolor where they play essential roles in sporulation. Wbl proteins contain around 100 amino acids. They are characterized by four conserved cysteine residues (18) that can act as ligands for iron (19), a tryptophan/glycine-rich motif, predicted to form a β-turn, and positively charged amino acids at their C termini (18) that match a sequence (RKPRGRPRKR) of a peptide that binds to AT-rich DNA sequences (AT-hook) (20). The AT-hook domain is found in eukaryotic non-histone chromosomal proteins (HMGAs) that have roles in chromatin architecture and transcriptional regulation (21). The cysteine motif resembles those known to coordinate redox-active Fe-S clusters. Indeed, many laboratories have demonstrated subpopulations of Fe-S clusters in Wbl proteins purified aerobically, often under denaturing conditions (19, 22–25). These preparations can be used to reconstitute iron-sulfur clusters; however, proteins assembled in this way contain substoichiometric concentrations of iron (19, 22, 23, 26). To our knowledge, there is only one report describing anaerobic purification of a soluble Wbl protein, presumably in its native form (27). Anaerobically purified WhiD contains a 4Fe-4S cluster having about 4 mol of iron per monomer (>70% occupancy). Mutagenesis experiments have shown that the conserved cysteines are needed for the biological functions of WhiB3 (25), WhiB4 (26), WhiD (19), WhiB2 (28), and the WhiB homolog of mycobacteriophage TM4 (22). However, the possibility that these mutant proteins are unstable has not been systematically addressed (except in one case (28)).

Although genetic and microarray studies suggest that Wbl proteins function as transcription regulators (7, 29), some members of this family may provide protein-disulfide reductase (23) or chaperone (30) activities. Direct biochemical evidence (transcriptional run off experiments) has assigned transcriptional regulatory functions to WhiB proteins, including whiB1 (31, 32), a repressor, and whiB7, an activator (33). In vitro transcriptional run-off studies have shown that the M. smegmatis WhiB7 protein is a redox-sensitive transcriptional activator of its own promoter (33). The whiB7 promoter, conserved across mycobacteria and other Actinobacteria, includes an AT-rich motif directly upstream of its −35 hexamer that is targeted by WhiB7 to promote transcription (9, 33). AT-rich motifs are also found upstream of other promoters in the whiB7 regulon (9) and are necessary for in vitro transcriptional activation (33). WhiB3 or WhiB7 function depends on their direct interactions with the vegetative σ factor RpoV (33, 34), and at least one Streptomyces σ factor gene encodes an N-terminal extension homologous to Wbl proteins (35).

**WhiB7 Activates Species-specific Drug Resistance**

Here, we show that WhiB7-mediated multidrug resistance spectra in Streptomyces lividans (closely related to S. coelicolor having an identical whiB7 sequence), M. smegmatis, and R. jostii are dependent on genome-specific resistance determinants and that WhiB7 activity requires the consensus sequence motifs of the Wbl protein family as well as its distinctive AT-hook DNA binding domain. In addition, the Streptomyces WhiB7 protein was, for the first time, anaerobically purified as dimers that coordinated a fully reduced, oxygen-sensitive Fe-S cluster.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Growth Conditions, and Chemicals**

Strains and plasmids used in this study are described in Table 1. The sequences of the oligonucleotides used are available upon request. *M. smegmatis* was routinely grown at 37 °C in Middlebrook 7H9 broth (Difco) supplemented with 10% Middlebrook albumin/dextrose/catalase (Difco), 0.5% glycerol, and 0.05% (v/v) tyloxapol or on Middlebrook 7H10 agar plates (Difco) supplemented with 10% (v/v) oleic acid albumin/dextrose/catalase (Difco). *R. jostii* RH1 and *S. lividans* were grown at 30 °C in NE medium (36). *S. lividans* spores were prepared as described elsewhere (37) and stored at −20 °C. Escherichia coli was grown at 37 °C in LB broth or on LB agar plates. Plasmids were maintained in *E. coli* with appropriate antibiotics for selection (100 μg/ml ampicillin and 20 μg/ml kanamycin). For the selection of resistance markers in *M. smegmatis* and *R. jostii*, hygromycin (50 μg/ml) or kanamycin (30 μg/ml) and apramycin (50 μg/ml), respectively, were added to the cultures. Antibiotics and chemicals used in sensitivity tests were obtained from Sigma.

**Plasmid Construction**

DNA manipulations were carried out using standard techniques (38). *E. coli*, *M. smegmatis*, and *R. jostii* were transformed by electroporation with a Gene Pulser Xcell™ (Bio-Rad).

*In Trans Expression of whiB7 Genes in M. smegmatis*—The whiB7 genes from *M. smegmatis* mc²155 (MSMEG_1953), *M. tuberculosis* H37Rv (rv3197a), *S. coelicolor* A3(2) (SCO5190), and *R. jostii* RH1 (ro06383) were amplified from genomic DNA, cloned into pGEM-T Easy (Promega), and their sequences verified. The engineered DNA fragments were then excised and cloned into the EcoRI and HindIII sites of pMV361. Plasmids were transformed into *M. smegmatis* mc²155 or its derived ΔwhiB7 strain.

*In Trans Expression of whiB7 Genes in R. jostii*—DNA fragments containing whiB7 genes from *M. smegmatis, M. tuberculosis, S. coelicolor*, and *R. jostii* RH1a were excised from plasmids pMV361: B7SM, pMV361: B7TR, pMV361: B7ST, and pMV361: B7RH, by MfeI/HindIII digestions and ligated into the multiple cloning site of vector pTIP-QC1, previously digested with EcoRI and HindIII. This vector contained a thiostrepton-inducible promoter (tipA). The obtained plasmids were transformed into *R. jostii* RH1 ΔwhiB7.

*N-terminal Truncations of the whiB7ST Gene*—Truncated forms of the whiB7 gene from *S. coelicolor* were constructed from pMV361: B7ST. Two truncated forms were generated at nucleotide position 28 (valine; Val-10) and at nucleotide posi-
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**TABLE 1**
Plasmids and strains used in this study

| Description                                      | Ref. |
|--------------------------------------------------|------|
| **Plasmids**                                     |      |
| pGEM-T Easy                                      | Promega |
| RF0120L2                                         | 44   |
| pUC-Hy                                           | 43   |
| RFMD15                                           |      |
| RFMD16                                           | This study |
| pvU85                                         | 40   |
| pYUB:whiB7ST                                     |      |
| pTIP-PCR1                                        | 46   |
| pTIP:B7SAT                                      | This study |
| pTIP:B7RH                                       | This study |
| pTIP:B7TB                                       | This study |
| pMV361                                          | 80   |
| pMV261                                          | 80   |
| pSUM36                                          | 45   |
| pMV361:B7SAT                                     | This study |
| pMV361:B7RH                                     | This study |
| pMV361:B7TB                                     | This study |
| pMV361:B7SM                                     | This study |
| pMV361:B7ST                                     | This study |
| pLN5                                             |      |
| pLN6                                             |      |
| pLN7                                             |      |
| pLN9                                             |      |
| pLN10                                            |      |
| pMV361 expressing whiB7ST                       | This study |
| pMV361 expressing whiB7RH                       | This study |
| pMV361 expressing whiB7TB                       | This study |
| pMV361 expressing whiB7SM                       | This study |
| pMV361 expressing C-terminal His-tagged whiB7ST | This study |
| pMV361 expressing C-terminal His-tagged whiB7RH | This study |
| pMV361 expressing C-terminal His-tagged whiB7TB | This study |
| pMV361 expressing C-terminal His-tagged whiB7SM | This study |
| pMV361 expressing structural tapR                | This study |
| pMV361 expressing erm(37) from Pcrab              | This study |
| pAC49                                            | 81   |
| pSUM36 expressing tapR from its native promoter  |      |
| pSUM36 expressing erm(37) from its native promoter| This study |
| pET19b                                           | This study |
| pBOO-1                                           | Novagen |
| whiB7ST cloned to Ndel/BamHI sites of PET19b     | This study |
| prKGC                                            | 39   |
| Plasmid with isc genes; TetR                     |      |

| **Strains**                                     |      |
|--------------------------------------------------|------|
| 1326                                             | 7    |
| 1326/ΔwhiB7 (RM1)                                | 7    |
| RHA1                                             | 83   |
| RHAlΔwhiB7                                       | This study |
| mc/6 Wild type M. smegmatis mc/6 (ATCC607) strain| 42   |
| mc/6/ΔwhiB7                                      | This study |
| mc/155                                           | 42   |
| mc/155/ΔwhiB7                                    | This study |
| 8x2l                                              |      |

**Mutagenesis of C-terminal His-tagged whiB7ST**—The whiB7ST gene was PCR-amplified from pB001 (pET19b: whiB7ST, see below) eliminating the whiB7ST stop codon. The DNA fragment was cloned into the Ndel and BglII sites of plasmid pET30b (Novagen). The entire whiB7ST gene fused to a C-terminal hexahistidine tag was amplified and subcloned into the PstI and Clal sites of pMV261. Five cysteine-motif mutated alleles of whiB7ST (C49S, C72S, C75S, C81S, and C72S/C75S) were constructed by site-directed mutagenesis of the C-terminal His-tagged whiB7ST gene cloned in pMV261 using QuikChange Lightning site-directed mutagenesis kit (Stratagene). The presence of all mutant alleles was confirmed by DNA sequencing. The resulting plasmids, both wild type and cysteine mutant constructs, were electroporated into M. smegmatis mc/155 ΔwhiB7.

**Cloning of His-whiB7ST in E. coli**—The whiB7ST gene was amplified from S. coelicolor A3(2) genomic DNA. The DNA
fragment was cloned into the NdeI and BamHI sites of pET19b (Novagen), introducing an N-terminal His tag. The new plasmid (pBOO-1) was co-transformed with plasmid pRKISC, harboring genes required for assembly of iron-sulfur clusters (isc) (39), into E. coli BL21(DE3) for expression of WhiB7ST.

**Strain Constructions**

The *S. lividans* whiB7 mutant has been reported previously (7). Replacement of whiB7 (*MSMEG_1953*) by a hygromycin resistance gene in *M. smegmatis* mc^6^ was performed by specialized transduction (40). The whiB7-5' (660 bp) and -3' (735 bp) flanking regions were PCR-amplified and inserted on either side of the resistance marker gene in plasmid pYUB854. Hygromycin-resistant colonies were analyzed, and the replacement was confirmed by recovering the mutant locus by PCR using primers flanking the allelic exchange substrate. The mutant DNA generated a larger PCR fragment corresponding to the targeted site in the wild type host strain. Replacement of the gene was confirmed by PCR as described above (data not shown). The hygromycin-resistant colonies were analyzed, and the replacement was confirmed by recovering the mutant locus by PCR using primers flanking the allelic exchange substrate. The mutant DNA generated a larger PCR fragment corresponding to the targeted site in the wild type host strain. Replacement of the gene was confirmed by PCR as described above (data not shown). The hygromycin-resistant colonies were analyzed, and the replacement was confirmed by recovering the mutant locus by PCR using primers flanking the allelic exchange substrate. The mutant DNA generated a larger PCR fragment corresponding to the targeted site in the wild type host strain. Replacement of the gene was confirmed by PCR as described above (data not shown).

Replacement of the whiB7 gene in *R. jostii* RHA1 (roo6338) by a apramycin resistance cassette was done as described previously (43). Briefly, in a first step the chloramphenicol resistance gene of fosmid RF00120122 was replaced by a hygromycin resistance gene. This fosmid harbors a 34.2-kb insert of genomic RHA1 DNA containing whiB7 (44). In a second step, the whiB7 gene was replaced by an apramycin resistance cassette, yielding the RFMD16 fosmid. RFMD16 was conjugated into RHA1 cells. Allelic exchange between the fosmid and the chromosome resulted in replacement of whiB7 with an apramycin resistance cassette. To confirm replacement of the whiB7 gene in *R. jostii*, apramycin-resistant and hygromycin-sensitive double crossover ex-conjugant colonies were selected and analyzed by PCR using combinations of primers homologous to the whiB7 gene, flanking the replacement locus and within the apramycin resistance gene cassette. In the mutant, both 5' and 3' junctions of the replacement locus were verified using primers for flanking regions and those within the apramycin resistance gene cassette. Substitution of whiB7 with the apramycin resistance gene cassette was further confirmed by Southern blot analysis. Genomic DNA (500 ng) of the whiB7 mutant and the wild type *R. jostii* RHA1 was digested with EcoRI and probed with digoxigenin-labeled 1.45-kb PCR fragment amplified from the fosmid RF00120I22. The mutant and the wild type strains generated the expected hybridization signals (data not shown).

For homologous and heterologous complementation experiments in *M. smegmatis*, wild type mc^155 and whiB7 mutant strains were transformed with the integrative plasmid pMV361 harboring the whiB7 genes from *M. tuberculosis*, *M. smegmatis*, *R. jostii*, or *S. lividans* under control of the constitutive mycobacterial *hsp* promoter. For *whiB7*-dependent drug resistance experiments, the *erm* (37) and *tap* genes from *M. tuberculosis* and the *tap* gene from *Mycobacterium fortuitum* were cloned under the control of their native promoters (including 500–700 bp upstream of the annotated start codon of the gene) into a promoter-less site of the pSUM36 vector (45), or under the control of the mycobacterial *hsp* constitutive promoter of the pMV361 vector (cloning the functional gene from the annotated start codon). Constructs were introduced into *M. smegmatis* mc^155 and corresponding whiB7 mutant. For complementation experiments in *R. jostii* RHA1, whiB7 mutant strains were transformed with the replicative plasmid pTIP-QC1 (46) harboring the whiB7 genes from *M. tuberculosis*, *M. smegmatis*, *R. jostii*, or *S. lividans* under the control of the thiostrepton-inducible *tipA* promoter.

**Western Blot Analyses of C-terminal His-tagged WhiB7 Mutants**

Plasmids pMV261 harboring wild type and cysteine mutant constructs of C-terminal His-tagged whiB7ST were transformed into the mc^155 whiB7 mutant and grown in 7H9 until the cell density reached an A_600 of ~1. Cultures (1 ml) were then subjected to a 45 °C heat shock treatment for 30 min in a water bath. Cells were chilled on ice, harvested by centrifugation at 4,000 rpm for 15 min, resuspended in 100 μl of SDS loading buffer, boiled at 95 °C for 10 min, and placed for 1 min on ice. The mixture was centrifuged to pellet the insoluble materials, and 3 μl of the sample supernatant was loaded onto a 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad) at 12 V for 75 min. Transfer Buffer was composed of 25 mM Tris, 190 mM glycine, and 20% methanol at pH 8.5. Following transfer to a nitrocellulose membrane, the membrane was blocked overnight at 4 °C with Blocking Buffer for fluorescent Western blotting (MB-070, Rockland Immunochemicals). After three washing steps of 20 min each in PBS containing 0.05% Tween 80, the blot was incubated for 3 h in the primary antibody solution composed of a mouse anti-His-tag antibody (catalog no. 200-301-382, Rockland Immunochemicals) at 0.5 μg/ml in Blocking Buffer containing 0.05% Tween 80. The blot was washed three times for 20 min with PBS plus 0.05% Tween 80, incubated for 3 h with the secondary antibody (goat anti-mouse IgG F(c) Antibody DyLight™ 680-conjugated, catalog no. 610-144-003, Rockland Immunochemicals), diluted to 0.2 μg/ml in Blocking Buffer with 0.05% Tween 80, and then washed three times for 20 min each with PBS plus 0.05% Tween 80. The presence of the hexahistidine epitope on the fusion WhiB7ST was visualized using a Li-Cor Odyssey Fluorescent Imager.
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WhiB7ST Expression, Purification, and Characterization

For expression of WhiB7ST, electro competent E. coli BL21(DE3) cells were co-transformed with plasmids pB00-1 and pRKISC, harboring isc genes to promote iron-sulfur cluster assembly (39). Transformants were grown on LB agar containing ampicillin (100 μg/ml) and tetracycline (20 μg/ml). Single colonies of transformants were inoculated into LB broth containing antibiotics for plasmid maintenance and grown overnight at 37 °C and 200 rpm. The starter culture was diluted 200-fold into 1 liter of LB media containing 100 μg/ml ammonia iron(III) citrate and selective antibiotics and grown at 37 °C with shaking until they reached an A600 of ~0.5. Cultures were then transferred to a water bath, and the temperature increased to 42 °C to induce expression of heat shock chaperone proteins. After 30 min, culture temperature was quickly lowered to 21 °C, and expression of WhiB7ST was induced using 0.1 mM isopropyl 1-thio-β-d-galactopyranoside. After overnight expression (16–18 h), cells were harvested by centrifugation at 4,000 rpm for 15 min and frozen at −80 °C. For purification, frozen cells were gently thawed at 4 °C overnight and then resuspended in Lysis Buffer (50 mM Tris/HCl, pH 7.6, plus 1 mg of DNase I (Roche Applied Science), 10 μl of 1 M CaCl2, and 10 μl of 1 M MgCl2 per 10 ml of buffer) at a ratio of 10 ml of Lysis Buffer per 1 liter of the original culture. Resuspended cells were lysed by passage through a pressure homogenizer at 10,000 p.s.i. until a clear homogenate was observed, transferred to an ultracentrifuge holder (Varian, Walnut Creek, CA). Samples (protein concentration ~15 μM) were assayed in 20 mM MOPS, 80 mM NaCl, pH 7.6 buffer. A molecular mass of 16 kDa for the N-terminal His-tagged WhiB7ST protein was used to calculate molar concentrations. Protein concentrations were determined using the Bradford assay with bovine serum albumin as a standard. Acid-labile sulfur content of samples was determined colorimetrically using the N,N-dimethylparaphenylenediamine assay (48). Iron content was determined using the Ferene S assay (49).

Drug Sensitivity Assays

The MICs of the compounds were determined using either resazurin or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (50). Briefly, serial 2-fold dilutions of the compounds were assayed in NE medium. The initial inoculum for M. smegmatis and R. jostii was 105 cells/ml; 106 spores/ml were used for S. lividans. Sensitivity dilution end points for M. smegmatis, R. jostii, and S. lividans were recorded at 96, 48 (or 72), or 24 h, respectively. The MIC value was defined as the lowest drug concentration that prevented a change in dye color. For some compounds, values were also confirmed in NE agar by a conventional antibiotic-containing disc assay (Pasteur Diagnostics) or using E-test® strips (AB Biodisks) and scored after 48–72 h of growth at 37 °C. For complementation assays, fold changes in the sensitivity of a given engineered strain are expressed with respect to the appropriate control strain. In the case of R. jostii, genes cloned under the control of the tipA promoter were induced with thiostrepton (0.5 μg/ml) for 1 h at 30 °C. Cultures were then added to 2-fold serial dilutions of erythromycin and tetracycline at a 1:1 ratio. Experiments were done in triplicate, each from three independent samples.

4 The abbreviation used is: Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
RESULTS

Inactivation of *whiB7* Generates Species-specific Drug Sensitivity Profiles in Actinobacteria—A representative collection of 38 drugs and chemicals was used to analyze the resistance profiles and possible physiological roles of the *whiB7* gene using mutants constructed in different Actinobacteria. Orthologous *whiB7* genes were disrupted in *M. smegmatis*, *R. jostii*, and *S. lividans*. Chemicals were selected to specifically inhibit different cellular processes such as cell wall assembly, DNA replication, transcription, translation, or redox balance. Quantitative analysis showed that the *whiB7* disruptions generated a unique sensitivity pattern in each of the three species (Fig. 1). All three *whiB7* mutants exhibited increased sensitivities to various antibiotics targeting protein biosynthesis. Interestingly, the increase in macrolide (azithromycin, clarithromycin, erythromycin, roxithromycin, and spiramycin) sensitivity caused by the mutation varied dramatically from 4- to 512-fold, depending on the species. Similarly, but to a lesser extent, mutants became more sensitive to other protein synthesis-targeting compounds such as aminoglycosides, tetracycline, lincomycin, fusidic acid, and phenicols (Fig. 1).

*R. jostii* and *S. lividans* *whiB7* mutants also displayed sensitivity to drugs targeting other essential cellular processes (Fig. 1), including compounds that disrupt fatty acid biosynthesis, DNA replication, and redox balance. Cerulenin, a fatty-acid synthase inhibitor, was 256 times more active against the *R. jostii* mutant. The mutation also affected sensitivity to compounds altering the intracellular oxidative state of these bacteria, such as the thiol oxidant diamide, the thiol reductant DTT, the thiol-specific alkylating agent mBBr, the reactive oxygen species generators menadione, or hydrogen peroxide. The *whiB7* mutant in *S. lividans* was slightly more sensitive to type 2 NADH dehydrogenase inhibitors (phenothiazines: chlorpromazine and thioridazine), suggesting a potential link between WhiB7 and respiration/redox stress. Finally, *whiB7* mutations in *R. jostii* and *S. lividans* caused 4-fold increased sensitivity to rifampin, an inhibitor of the RNA polymerase, whereas the mutation in *M. smegmatis* did not alter its rifampin sensitivity.

In summary, *whiB7* mutations led to multidrug sensitivity in Actinobacteria. Compounds targeting ribosome function showed the most dramatic effects, but increased sensitivities to inhibitors of other cellular processes were also observed. The spectrum and levels of sensitivity varied in different species of the actinobacterial phylum. This suggested that actinobacterial species each carry specific repertoires of *whiB7*-controlled drug resistance genes.

WhiB7 Proteins Are Functional in Heterologous Actinobacteria—WhiB7 proteins displayed a conserved core sequence (Fig. 2; their variable N termini are annotated) with characteristic residues, including cysteines that may bind iron-sulfur clusters, a glycine-tryptophan-rich region that predicts a unique turn in the protein conformation, and an AT-hook DNA-binding motif. These sequence motifs suggested a universal function and regulatory features conserved in actinobacterial WhiB7 proteins. In principle, the fact that inactivation of the *whiB7* gene conferred distinct species-specific changes in resistance patterns could be due to divergent functional speci-

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**FIGURE 1.** Visual display of the *whiB7* mutant sensitivity fingerprint in different Actinobacteria. An increase in the size of the dot corresponds to an increase in the sensitivity of the *whiB7* mutant to a given compound compared with the parental strain based on MIC determinations over a range of 2-fold serial drug dilutions. The scale is indicated at the bottom of the figure.
WhiB Activates Species-specific Drug Resistance

| Strains* (host/vector/cloned gene) | MIC† (μg/ml) of M. smegmatis strains |
|-----------------------------------|-------------------------------------|
| | ERY | Fold change‡ | SPT | Fold change‡ | TET | Fold change‡ |
| Parental/pMV361/ | 32–64 | 16 | 16 | 0.125 | |
| ∆whiB7/pMV361/ | 2 | ↑ 16–32 | 1 | ↑ 16 | 0.015 | ↑ 8 |
| whiB7 expression | | | | | | |
| ∆whiB7/pMV361/whiB7SM | 32 | 16 | 16 | 0.125 | 8 |
| ∆whiB7/pMV361/whiB7RH | 8–16 | 4–8 | 8–16 | 0.06 | 4 |
| ∆whiB7/pMV361/whiB7ST | 64 | 32 | 16 | 0.125 | 8 |
| ∆whiB7/pMV361/whiB7ST | 4–8 | 2–4 | 16 | 0.06 | 4 |
| erm(37) expression | | | | | | |
| ∆whiB7/pMV361/erm(37) | 128 | 64 | 1 | 0 | 0.015 | 0 |
| Parental/pSUM36/erm(37) | 128 | 2–4 | 16 | 0.125 | 0 |
| ∆whiB7/pSUM36/erm(37) | 2 | 0 | 1 | 0 | 0.015 | 0 |
| tap expression | | | | | | |
| ∆whiB7/pMV361/tapRH | 2 | 0 | 16 | 0.125 | 8 |
| Parental/pSUM36/tapRH | 2 | 0 | 32–64 | 2–4 | 0.25–0.5 | 4 |
| ∆whiB7/pSUM36/tapRH | 2 | 0 | 2 | 1–2 | 0.015 | 0 |
| Parental/pSUM36/tapRH | 64–128 | 2 | 16 | 4–8 | 32–64 |
| ∆whiB7/pSUM36/tapRH | 2 | 0 | 1 | 0.125 | 8 |

* Parental control host M. smegmatis (mc²155) or its ∆whiB7 derivative, carried pMV361 (hsp promoter to provide expression of cloned genes) or pSUM36 (without vector-encoded promoter).

† MICs were assayed over a range of 2-fold serial drug dilutions. ERY, erythromycin; SPT, spectinomycin; TET, tetracycline.

‡ Fold change in sensitivity compared with the control strain. The ∆whiB7 strain had increased sensitivity (↑) compared with the parental control. All other decreases in sensitivity generated by cloned genes are expressed relative to the corresponding host with empty vector.

...defined by specificities of genes in their hosts' whiB7 drug resistance regulons and not by divergent functional specificities of the corresponding WhiB7 proteins.

Expression of Heterologous whiB7-dependent Genes in M. smegmatis Defines Their Resistance Functions—In M. tuberculosis, overexpression and inactivation of whiB7 led to the identification of genes in the whiB7 regulon (7). The whiB7 regulon includes several genes associated with antibiotic resistance, such as tap (Rv1258c), an efflux pump that confers resistance to aminoglycosides and tetracyclines and is also involved in maintaining cellular homeostasis in the nonreplicative state (13), and erm(37) (Rv1988) (14), a ribosomal methyltransferase that confers macrolide resistance by modification of the 23 S rRNA. Genetic approaches and drug sensitivity assays were used to demonstrate a direct relation between their expression and whiB7 mutant phenotypes (Fig. 3). The erm(37) and tap genes from M. tuberculosis (tapRH) or M. fortuitum (tapRF), cloned downstream of hsp promoter (to provide for WhiB7-independent gene transcription), or their native promoters (WhiB7-dependent gene transcription) were analyzed in M. smegmatis wild type or whiB7 mutant backgrounds. Sensitivities of these strains to representative antibiotics affected by the whiB7 mutation in M. smegmatis, i.e. spectinomycin, erythromycin, and tetracycline, are shown in Table 2.
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TABLE 3

Drug sensitivity phenotype suppression of the whiB7 mutation by heterologous gene expression in R. jostii RHA1

| Strainsa (host/vector/gene) | MICb (µg/ml) of R. jostii strains | Fold changec | MICb (µg/ml) of R. jostii strains | Fold changec |
|-----------------------------|----------------------------------|-------------|----------------------------------|-------------|
|                            | ERY − THIO + THIO                |             | TET − THIO + THIO                |             |
| Parental/pTIP/−             | 2–4 2–4                          | 1           | 1–2 1–2                          | 1           |
| ΔwhiB7/pTIP/−               | 0.0625–0.125 0.0625–0.125        | 1           | 0.06 0.06                         | 1           |
| whiB7 complementation       |                                  |             |                                  |             |
| ΔwhiB7/pTIP/whiB7mut        | 0.125–0.5 2–4                    | >4          | 0.06–0.125 0.5–1                 | >4          |
| ΔwhiB7/pTIP/whiB7mut        | 0.25 2–4                         | >8          | 0.125 2                           | 8           |
| ΔwhiB7/pTIP/whiB7erm        | 0.25 8 32                        | 1           | 0.06–0.125 1                     | >8          |
| ΔwhiB7/pTIP/whiB7erm        | 0.125 2 16                       | 1           | 0.125 2                           | 16          |

a MICs were assayed over a range of 2-fold serial drug dilutions. Genes were cloned under the control of the thioestrepton-inducible tipA promoter and susceptibility assayed with and without induction. ERY, erythromycin; TET, tetracycline; THIO, thioestrepton.
b Control strains contain empty pTIP vector.
c Fold change in susceptibility of the ΔwhiB7 mutant after thioestrepton induction.

Conserved Sequence Motifs Are Required for WhiB7 Activity in Vivo—The WhiB7 protein from S. lividans (WhiB7St) was mutagenized to define requirements for the in vivo function of the predicted N-terminal amino acids, the conserved cysteines, the glycine/tryptophan-rich region, and the AT-hook DNA-binding motifs (Fig. 2). Two shorter versions of the annotated WhiB7St protein, deleting 9 and 28 amino acids from the annotated N terminus (Val-10 and Pro-29, respectively) were cloned under the control of the hsp60 constitutive promoter. Both forms restored wild type resistance levels to erythromycin and spectinomycin when introduced to a whiB7 mutant in M. smegmatis (Table 4). However, complementation was more efficient in cultures expressing the shorter protein, Pro-29; resistance levels were consistently restored 24 h earlier compared with Val-10 or protein corresponding to the annotated start site. The annotated full-length WhiB7St protein has a calculated molecular mass of 17 kDa, and the Pro-29 form is 14 kDa. Pro-29 was closely linked to an in-frame TTG potential start codon (annotated Leu-27) and a potential upstream ribosome-binding site.

To test whether the AT-hook motif was required for WhiB7St activity, clones carrying four mutated alleles were assayed for their abilities to restore resistance to three antibiotics: erythromycin, spectinomycin, or chloramphenicol (Table 4). L2, encoding a protein lacking the five C-terminal amino acids adjacent to the AT-hook motif, restored wild type resistance levels, suggesting that this region of the C terminus was not required for WhiB7St activity. In contrast, the L1 form, lacking both the AT-hook and the five C-terminal amino acids, and L4, lacking the AT-hook but retaining the five C-terminal amino acids, did not complement the whiB7 mutation. The double point mutations in L5, in which the two prolines of the AT-hook were mutated to alanine (P106A/P118A; residues needed to maintain a trans-configuration in the AT-hook for proper functionality (51, 52)), provided decreased complementing activity. These data suggested that the AT-hook DNA binding domain was required for full WhiB7St activity.

Another WbI family sequence signature is a tryptophan/glycine-rich motif (Fig. 2). A mutant form of WhiB7St (L5N) in which the motif was mutated (deletion of eight amino acids; WGTVWGVEL) was unable to restore antibiotic resistance (Table 4), suggesting that it is required for WhiB7 function. As this motif is conserved in all Whi-like proteins, it probably provides a similar, essential role throughout the WbI family.
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To evaluate the requirement of the four conserved cysteine residues for WhiB7ST function, five alleles of whiB7ST were constructed in which triplets encoding each individual or the two most closely linked cysteines were mutated to serine (C49S, C72S, C75S, and C72S/C75S) (Table 4). Mutant strains had obvious defects in activating antibiotic resistance genes (erythromycin, chloramphenicol, and spectinomycin) in an M. smegmatis whiB7 mutant background. Interestingly, the C49S allele partially complemented the spectinomycin sensitivity profile (MICs: wild type, >30 μg/ml; whiB7 mutant, <5 μg/ml; and C49S, 10 μg/ml), suggesting that this particular WhiB7ST mutation retained partial activity. These data suggested that at least three of the four conserved cysteines were essential for WhiB7 activity.

The conserved cysteines of WhiB-like proteins are generally needed to coordinate their iron-sulfur clusters (19, 22, 23, 26–28, 53–55) that can provide altered structures and functions to transcriptional regulatory proteins (19, 22, 23, 26–28, 53–56). Western blot analyses were performed using C-terminal His-tagged WhiB7ST proteins to determine whether the lack of complementation by cysteine-mutated forms of the WhiB7ST protein could be attributed to reduced expression or increased degradation of the mutant proteins (Fig. 4). The addition of a His6 tag at the C terminus of the WhiB7ST protein did not alter its biological activity; His-tagged WhiB7ST cysteine mutants had similar activities as corresponding untagged proteins, i.e. the only mutant protein with detectable activity was C49S (data not shown). In Western blot analyses of the parental strain, WhiB7ST migrated roughly as predicted by its sequence (~17 kDa). WhiB7ST C49S co-migrated with wild type protein, although the C72S, C75S, C81S and C72S/C75S forms migrated faster (~14 kDa) (Fig. 4). Interestingly, migration patterns correlated with in vivo activity, suggesting unstable iron-sulfur clusters were present in the holoprotein when cysteines at positions 72, 75, and 81 were replaced by serines, leading to cleavage of the protein. In contrast, the C49S mutation might still allow weaker stabilization of the holoprotein providing some in vivo activity. Although most iron-sulfur clusters are bound by four cysteines, in some cases the protein activity can be retained when other amino acids replace one of the cysteines (57, 58).

Table 4: Genetic elucidation of the in vivo role of the N-terminal region, conserved cysteines, the glycine-tryptophan-rich region, and the DNA-binding AT-hook motif of the WhiB7ST protein

| Strains (host/vector/gene) | Mutation | ERY | CHL | SPT |
|----------------------------|----------|-----|-----|-----|
| Parental None              | None     | >8  | 16  | >30 |
| ΔwhiB7/PMV361/−             | None     | 4   | <4  | <5  |
| ΔwhiB7/PMV361/B7ST         | None     | >8  | 32  | >30 |
| ΔwhiB7/PMV361/V10          | N-terminal | >8  | 32  | >30 |
| ΔwhiB7/PMV361/P29          | N-terminal | >8  | 32  | >30 |
| ΔwhiB7/PMV361/LN1          | AT hook  | 6   | <4  | <5  |
| ΔwhiB7/PMV361/LN2          | AT hook  | >8  | 16  | >30 |
| ΔwhiB7/PMV361/LN3          | AT hook  | 8   | 8   | >30 |
| ΔwhiB7/PMV361/LN4          | AT hook  | 6   | <4  | <5  |
| ΔwhiB7/PMV361/LN5          | GW-rich region | 4   | <4  | <5  |
| ΔwhiB7/PMV361/LN6          | C49S     | 4   | 4   | 10  |
| ΔwhiB7/PMV361/LN7          | C72S     | 4   | <4  | <5  |
| ΔwhiB7/PMV361/LN8          | C75S     | 4   | <4  | <5  |
| ΔwhiB7/PMV361/LN9          | C81S     | 4   | <4  | <5  |
| ΔwhiB7/PMV361/LN10         | C72S/C75S | 4   | <4  | <5  |

* MICs were determined using E-test® (AB Biodisk). The parental strain, mc²6, carried pMV361-derived vectors, some containing whiB7 mutant genes from S. coelicolor (whiB7*): V10, nine amino acids deleted from the N terminus; P29, 28 amino acids deleted from the N terminus; LN1, lacking both the AT-hook and the last five C-terminal amino acids; LN2, lacking the five C-terminal amino acids; LN3, two prolines mutated to alanine (P106A and P118A); LN4, without the AT-hook but retaining the last five C-terminal amino acids; LN5, the tryptophan/glycine-rich motif (WGVWGGEL) deleted; and a series of mutants with the conserved cysteines mutated to serine: C49S (LN6), C72S (LN7), C75S (LN8), C81S (LN9), and C72S/C75S (LN10), ERY, erythromycin; CHL, chloramphenicol; SPT, spectinomycin.
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### A.

![Graph showing absorption (Abs) at different wavelengths (nm)]

- **t = 0 hours**
- **t = 15 hours**

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### B.

1. **Protein Untreated** (Red)
2. **Protein: DTH 1:10** 1 hour (Green)
3. **Protein: [Fe(CN)]₆⁻³ 1:10** 1 hour (Pink)

![Graph showing absorption (Abs) at different wavelengths (nm)]

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### C.

![Graph showing elution volume (mL) vs. absorbance (mAU)]

- **UV1_280nm**
- **UV3_413nm**

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[Graphs and data for absorption and elution volume analysis provided.]
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The oligomeric state of the anaerobically purified WhiB7ST was also determined under anaerobic conditions using size exclusion chromatography. The elution volume of WhiB7ST corresponded to a molecular mass of 34 kDa (Fig. 5C). Because the theoretical molecular mass of the N-terminal His-tagged WhiB7ST is 16 kDa, and provided that the protein is globular in character, these results indicated that N-terminal His-tagged WhiB7ST protein was a dimer when purified anaerobically.

**DISCUSSION**

Multiple Wbl genes are a unique feature of actinobacterial genomes, one of the largest and most diverse bacterial phyla (29). These genes may have evolved independently to provide specific functions in Actinobacteria having different shapes, developmental systems, habitats, and metabolisms and have also been adopted by actinobacterial plasmids and phages (29). Most whiB paralogs are not found in all genomes, reflecting evolutionary divergence of genes having specialized functions. In contrast, whiB1, whiB2, whiB3, whiB4, and whiB7 are ubiquitous (29), implying that they are the foundations for common core phenotypes that are retained throughout this bacterial taxon. Retention of whiB7 paralogs suggests that genes in their regulons provide selective advantage under stress conditions commonly experienced by Actinobacteria. Our results demonstrate that native whiB7 genes are required for expression of resistance determinants encoded by their genomes (represented by *erm* (37) and *tap*) and that whiB7 genes are also able to activate drug resistance genes present in heterologous Actinobacteria. This supports the concept that many antibiotics induce a common physiological response that activates WhiB7 in all Actinobacteria (9). Here, we used genetic and biochemical tools to investigate the iron-sulfur cluster, a redox sensor, as a probable antibiotic-induced effector of changes in WhiB7 structure and activity and then characterized resistance genes under whiB7 control.

Experiments showing that whiB7 genes function effectively in heterologous actinobacterial hosts suggested the presence of conserved sensor and effector regulatory partners. Extending what has been reported for WhiB3TS (34), our studies of WhiB7SM (33) have shown that it binds to a conserved amino acid sequence in the primary σ factor found in all Actinobacteria. It is noteworthy that the promoter region of *whiB7, tap, erm*, and *eis* genes from *M. tuberculosis* includes a conserved WhiB7 AT-rich binding site upstream of the −35 promoter hexamer (9). WhiB7 functions as a transcriptional activator, and the requirement for the AT-hook module suggests conserved target promoter sequence recognition motifs serving to control genes that provide multidrug resistance. Our genetic approach provided in vivo evidence supporting this hypothesis (Fig. 3). Iron-sulfur clusters in WhiB7 may respond to changes in redox balance in a way that localizes RNA polymerase to promoters containing AT-rich motifs and thereby activates transcription (33, 34). Although WhiB7 recognizes an AT-rich motif, recognition sequences for other Wbl proteins are not defined. In the case of WhiB7, there is evidence that other regulators are involved (9, 60).

To characterize more homogeneous, biologically relevant forms of WhiB7, we studied anaerobically purified WhiB7. Previous studies have typically utilized Wbl proteins that were isolated as inclusion bodies, solubilized in urea, purified under aerobic conditions, and reconstituted in vitro. The absorption curve of anaerobically purified WhiB7 from *S. lividans* suggested that it could coordinate a 4Fe-4S cluster (Fig. 5A). This spectrum was indistinguishable from anaerobically purified WhiD from *S. coelicolor*, reported to contain a 4Fe-4S cluster (27). It also lacked the characteristic broad shoulders (424, 460, and 560–580 nm) of aerobically purified WhiB7 from *M. tuberculosis* and WhiD from *S. coelicolor* proteins reported to have 2Fe-2S clusters (19, 23). However, chemical determination of the iron content in our anaerobically purified WhiB7ST demonstrated the presence of only two atoms each of iron and sulfur atoms per protein monomer. The apparent inconsistencies between these data might reflect a mixed population of apo- and holo-forms of the protein, containing only about 50% in the 4Fe-4S form. It is not uncommon to observe low occupancy rates in Fe-S cluster coordinating proteins, including Wbl proteins. This has also been reported for other Fe-S cluster coordinating proteins; *Layer et al.* (61) reported that anaerobically purified HemN protein from *E. coli* coordinated a 4Fe-4S cluster with incomplete iron incorporation (2 mol of iron/mol of HemN). Alternatively, it is conceivable that dimeric WhiB7 harbors a single 4Fe-4S cluster. Additional biochemical studies are required to test this hypothesis. Finally, the strong reductant sodium dithionite was unable to further reduce WhiB7ST (Fig. 5B), suggesting that the cluster is fully reduced as anaerobically purified.

Replacement of cysteine with serine destabilizes iron-sulfur clusters, and therefore cysteine to serine mutations have been employed to probe whether particular cysteine residues participate as iron-sulfur ligands (62). Intuitively, the three most adjacent cysteines in WhiB7ST (Cys-72, Cys-75, and Cys-81) should form closer contacts with iron atoms, whereas the distal cysteine (Cys-49) might form a loop to stabilize the iron-sulfur pocket. Sequence analyses showed that these three conserved cysteines are invariant among Wbl protein sequences, whereas the first cysteine is occasionally replaced by aspartate (18, 19, 26, 28). Our mutagenesis data suggested that these three vicinal cysteines (Cys-72, Cys-75, and Cys-81) were essential for normal WhiB7ST activity, whereas the first cysteine (Cys-49) was

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**FIGURE 5. Absorbance spectra and oligomeric state of anaerobically purified WhiB7ST proteins.** A, samples were air-bubbled for 30 s and remained exposed to the air for 15 h. The blue line represents anaerobic absorbance spectra (t = 0 h), red lines aerobic spectra (from 0 to 1 h), and black lines absorbance spectra from 1 to 15 h after oxygen exposure. Inset, a one-phase exponential decay curve was fitted and calculated a *T*<sub>2</sub>(413 nm) = 23.19 to 34.65 min (95% confident interval. *R*<sup>2</sup> = 0.9927) and *T*<sub>2</sub>(280/413) = 32.78 to 41.41 min (95% confident interval. *R*<sup>2</sup> = 0.9940). B, effect of the strong oxidant ferricyanide and reductant sodium dithionite on the absorption spectrum of anaerobically purified WhiB7ST proteins. Samples were exposed for 1 h to the redox agent at a molar protein/redox agent ratio of 1:10 and then desalted before recording the UV-visible spectra. C, anaerobic determination of the WhiB7 oligomeric state. Size exclusion chromatography was performed, and protein elution was followed at absorbance values at 280 and 413 nm. Inset, theoretical calculation of WhiB7 oligomeric size. The molecular mass for the N-terminal His-tagged WhiB7ST protein was ~16 kDa.
partially dispensable. Mutagenesis of corresponding cysteines in other Wbl proteins has suggested that they are essential for in vitro assembly of the iron-sulfur cluster (53, 55). In vivo, all four residues are essential for the function of WhiD (19); however, in other Wbl proteins, WhmD (28) and WhiBTM4 (22), their N-terminal conserved cysteines were not essential. Cysteine mutations at these essential sites probably led to inefficient assembly or increased release of Fe-S clusters that can cause instability of WhiB7 (33) and WhiBTM4 proteins (22). This could generate inactive forms of the intact apoprotein in vivo.

Unfolded proteins without disulfide bonds typically migrate more slowly on SDS-PAGE than their oxidized forms, an effect that can also be achieved by the inclusion of reducing agents such as mercaptoethanol (22, 28, 63–65) or cysteine mutations (22, 28, 63, 64). Because secondary structure cannot explain the faster migration of mutant proteins on our SDS-polyacrylamide gels, we conclude that the faster migrating peptides detected by antibodies against the C-terminal hexahistidine tag were likely to have been generated by cleavage near their N termini. Therefore, these data, as well as mutational studies of other Fe-S-containing proteins, suggest that the cysteine to serine (62) or cysteine to alanine mutations (33) might affect protein folding pathways or structural features that determine stability and function. Clarification of how these cysteine residues form the iron-sulfur cluster of Wbl proteins under reducing conditions and unfold in the presence of oxygen awaits three-dimensional structural determinations.

Iron-sulfur clusters can serve as sensors of iron and oxygen (56, 66, 67) that modulate the activities of transcriptional and translational regulatory proteins (68–70). In Mycobacterium, expression of whiB7 responds to diverse antibiotics, an effect amplified by the presence of a reducing agent in the medium (9). It is also induced by iron starvation (10), a stimulus that may explain activation early after macrophage infection (11). It is noteworthy here that the redox-responsive transcription regulator of antibiotic resistance and oxidative stress response in E. coli, SoxR, also contains an essential iron-sulfur cluster (71). SoxR is constitutively expressed at low levels in its reduced state. Reactive oxygen radicals or nitric oxide oxidize its iron-sulfur cluster, which generates a form of the protein that promotes expression of the transcription factor SoxS. SoxS activates genes providing for repair of DNA damage, removal of superoxide, and resistance to antibiotics. By analogy, WhiB7 or other WhiB-like proteins are likely to be modulated by altered oxidative states. The oxygen sensitivity of a 4Fe-4S cluster of the Streptomyces WhiD sporulation regulatory protein is dependent on four conserved cysteine residues; mutations of these cysteines resulted in sporulation defects (19). Oxygen limitation may be also a factor in triggering the Streptomyces sporulation program (72). M. tuberculosis entering the dormant state in host macrophages must adapt to low oxygen concentrations, which trigger metabolic shifts required for survival. These processes may result in many spore-like characteristics of inactive persistent mycobacterial cells, including enhanced antibiotic resistance (73). The N-terminal module containing the iron-sulfur cluster is linked to a conserved motif predicted to encode a β-turn and the AT-hook motif. Site-directed mutagenesis showed that both are necessary for WhiB7ST activity in vivo.

The role of AT-hooks in transcription regulation and chromosome architecture has been a focus of research in eukaryotes; prokaryotic regulatory proteins containing AT-hooks have also been identified (74–76). Often found as an auxiliary module within DNA-binding proteins or protein complexes, the AT-hook binds to the minor groove of AT-rich sequences and alters DNA conformation (20, 77). Although the AT-hook motif has no detectable secondary structure in solution, NMR studies have demonstrated that the two prolines are in the trans configuration (51, 52). This restricts the flexibility of the protein and creates a bend in solution (52) that may facilitate initial DNA binding. When these residues are mutated to alanine or when their position in the peptide motif is altered, the mutant peptide no longer binds to AT-rich DNA sequences in vitro (78); these mutant alleles lose their biological function in vivo (79). Exchanging corresponding prolines to alanines in WhiB7ST was therefore expected to weaken binding of the AT-hook to its DNA targets, and indeed our result showed that these mutant alleles were functionally impaired and could only provide low levels of antibiotic resistance in the whiB7 mutant. Taken together, these results suggested that the AT-hook DNA-binding motif facilitated binding of WhiB7ST to DNA targets and may have enhanced the activities of other transcription regulators or σ factors by altering protein-protein interactions or DNA conformation. Although other Wbl proteins may not contain a motif that has been demonstrated to bind DNA, the positively charged C terminus of other Wbl proteins is believed to provide a similar function (18, 22, 53).

The iron-sulfur cluster in SoxR is essential for transcription activation but is not required for the initial folding or to maintain its structure and DNA-binding affinity (71). It was suggested that the iron-sulfur activated form of SoxR functions in remodeling the soxR promoters such that they can form an “active” complex with RNA polymerase preceding transcriptional initiation (71). Similarly, most AT-hook proteins are also unstructured or marginally structured in solution and undergo a specific type of folding when bound to DNA targets or protein partners (77). Thus, WhiB7 proteins display common regulatory partners and control target genes that provide comparable multidrug resistance functions. Interactions with potential sensory regulatory partners, such as RNA polymerase σ factors, promoter-binding sites, reductases, etc., must also be conserved.

In addition, these results provide important information for structural elucidation of WhiB7 and other WhiB-like proteins, which might help develop novel antimycobacterial compounds. Such inhibitors might render pathogenic mycobacteria more sensitive to available repertoires of clinically approved antibiotics, which may widen chemotherapeutic options to treat mycobacterial diseases.

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