Mapping Essential Domains of *Mycobacterium smegmatis* WhmD: Insights into WhiB Structure and Function

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A growing body of evidence suggests that the WhiB-like proteins exclusive to the GC-rich actinomycete genera play significant roles in pathogenesis and cell division. Each of these proteins contains four invariant cysteine residues and a conserved helix-turn-helix motif. *whmD*, the *Mycobacterium smegmatis* homologue of *Streptomyces coelicolor* *whiB*, is essential in *M. smegmatis*, and the conditionally complemented mutant *M. smegmatis* 628-53 undergoes filamentation under nonpermissive conditions. To identify residues critical to WhmD function, we developed a cotransformation-based assay to screen for alleles that complement the filamentation phenotype of *M. smegmatis* 628-53 following inducer withdrawal. *Mycobacterium tuberculosis* *whiB*2 and *S. coelicolor* *whiB* complemented the defect in *M. smegmatis* 628-53, indicating that these genes are true functional orthologues of *whmD*. Deletion analysis suggested that the N-terminal 67 and C-terminal 12 amino acid residues are dispensable for activity. Site-directed mutagenesis indicated that three of the four conserved cysteine residues (*C*90, *C*93, and *C*99) and a conserved aspartate (*D*71) are essential. Mutations in a predicted loop glycine (*G*111) and an unstructured leucine (*L*116) were poorly tolerated. The region essential for WhmD activity encompasses 6 of the 10 residues conserved in all seven *M. tuberculosis* WhiBs, as well as in most members of the WhiB family identified thus far. WhmD structure was found to be sensitive to the presence of a reducing agent, suggesting that the cysteine residues are involved in coordinating a metal ion. Iron-specific staining strongly suggested that WhmD contains a bound iron atom. With this information, we have now begun to comprehend the functional significance of the conserved sequence and structural elements in this novel family of proteins.

*Streptomyces coelicolor*, a gram-positive, sporulating bacterium, is phylogenetically a close relative of *Mycobacterium tuberculosis*, with a similarly high GC content (65 to 70%). This organism follows a differentiation cycle in which young colonies send hyphal extensions into the agar and later-generation cells form white aerial hyphae which become pigmented and produce spores (4). Mutants which show an arrest in the development of mature spores and pigment formation remain white, and mutations leading to this phenotype have been shown to be involved in DNA binding (21). These proteins all possess a high overall hydrophilicity suggestive of a cytoplasmic location. Despite an overall negative charge, these proteins have positively charged regions near their carboxy termini (17) and are predicted to contain extensive α-helical structures with a central β-sheet region between the first and second α-helices (21). Analysis of the genome sequence (7) indicates that *M. tuberculosis* contains seven *whiB* homologues (*whiB*1 to *whiB*7), which show all the hallmark features of the members of the WhiB family. The presence of four cysteine residues suggests that these proteins may be sensitive to redox changes, perhaps through a bound metal atom or through direct sensitivity to oxidation via disulfide bond formation. A recent report demonstrated that the *Streptomyces* developmental protein WhiD, a member of the WhiB family, binds a [4Fe-4S] cluster (13) and that all four cysteine residues are essential for WhiD activity. A second recent article showed that the *whcE* gene of *Corynebacterium glutamicum*, also a member of the *whiB* family, is important for survival following heat and oxidative stress (14). Both these observations lend support to the hypothesis that the members of this family of proteins are likely to be associated with intracellular redox-sensing pathways.
Although the most C-terminal a-helix of the putative HTH-like domain contains a segment rich in basic residues likely to be involved in DNA binding (24), there are no published reports demonstrating the interaction of any of the WhiB family of proteins with DNA. In addition, the significance of the 10 conserved amino acid residues and the conserved predicted secondary structural elements in all the Whi-like proteins remains unknown.

WhmD (WhiB2 in *M. tuberculosis*), which by amino acid similarity is the closest *Mycobacterium smegmatis* orthologue of Streptomyces coelicolor WhiB, is encoded by *whmD*, an essential mycobacterial gene required for proper septation and cell division. In *M. smegmatis*, this gene could be disrupted only in the presence of a plasmid supplying *whmD in trans*. In a conditionally complemented system, on withdrawal of the inducer, the mutant exhibited irreproducible filamentous branched growth with diminished septum formation and aberrant septal placement (10). Computer algorithm-based secondary-structure analysis predicted that, like all WhiB-like proteins, WhmD is a putative membrane protein (10). Computer algorithm-based secondary-structure analysis predicted that, like all WhiB-like proteins, WhmD is a putative membrane protein (10).

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions.** *Escherichia coli* strain DH5α (*F' endA1 hsdR17 [F' proAB lacI*Δ (lacUZΔM15)] de3) (Novagen, Madison, WI) was used for cloning purposes. *E. coli* BL21(DE3) (*F ompT hsdS (rK*) (F *) gal dcm (DE3)) was used for protein expression was purchased from Novagen, Madison, WI. *M. smegmatis* mc2 615-12 was kindly provided by Bill Jacobs, Albert Einstein College of Medicine, New York, NY. *M. tuberculosis* CDC1551 genomic DNA was obtained from Colorado State University. The vector pET-22b (+) was purchased from Novagen, Madison, WI. *Luria-Bertani* (LB) broth and LB agar were used to culture *E. coli*. 7H9 broth and 7H10 agar from Difco Laboratories (Becton Dickinson) were supplemented with albumin dextrose complex (5 g/liter bovine serum albumin, 2 g/liter dextrose, 0.85 g/liter NaCl), 0.2% glycerol and were used for culturing mycobacteria. Both *E. coli* and mycobacteria were grown at 37°C with shaking at 200 rpm. The following antibiotics were added when necessary: ampicillin (200 µg/ml), kanamycin (50 µg/ml for *E. coli* and 15 µg/ml for mycobacteria), hygromycin (200 µg/ml for *E. coli* and 50 µg/ml for mycobacteria), apramycin (30 µg/ml), and zeocin (25 µg/ml for mycobacteria).

**DNA techniques.** Restriction enzymes and T4 DNA ligation were purchased from New England Biolabs (NEB), Beverly, MA, and Tag polymerase was purchased from Invitrogen Corporation, CA. *Pho* DNA polymerase was purchased from Stratagene, CA. The *Klenow* fragment of DNA polymerase was purchased from NEB. Protocols for DNA manipulations, including plasmid DNA preparation, restriction endonuclease digestion, agarose gel electrophoresis, isolation of DNA fragments, *E. coli* transformation, and transformation of *M. tuberculosis* were purchased from Stratagene, CA. The vector pET-22b (+) was purchased from Novagen, Madison, WI. *Luria-Bertani* (LB) broth and LB agar were used to culture *E. coli*. 7H9 broth and 7H10 agar from Difco Laboratories (Becton Dickinson) were supplemented with albumin dextrose complex (5 g/liter bovine serum albumin, 2 g/liter dextrose, 0.85 g/liter NaCl), 0.2% glycerol and were used for culturing mycobacteria. Both *E. coli* and mycobacteria were grown at 37°C with shaking at 200 rpm. The following antibiotics were added when necessary: ampicillin (200 µg/ml), kanamycin (50 µg/ml for *E. coli* and 15 µg/ml for mycobacteria), hygromycin (200 µg/ml for *E. coli* and 50 µg/ml for mycobacteria), apramycin (30 µg/ml), and zeocin (25 µg/ml for mycobacteria).

**Construction of plasmid pBP10 zeo and test alleles for complementation.** Plasmid pBP10 zeo was constructed by excising a 1.1-kb fragment from the vector pER10 (E. Rubin, unpublished data) using the enzymes EcoRI and XbaI, end-filling using the Klenow fragment of *E. coli* DNA polymerase, and inserting the resulting fragment into the EcoRV site of pBP10. Plasmid pBP10 containing 187 bp of its 5′ untranslated region (UTR) was amplified from *M. smegmatis* genomic DNA using the resulting PCR primers pBP10whmD-F (5′ AAAATTGACGAATCCTGCGC CTGAGAC 3′) and pBP10whmD-R (5′ GGAATTCTTGAATCTGCGC CTGACG 3′) and cloned at the PstI-SpeI sites of pBP10 zeo to generate pBP10 zeo whmD. The *S. coelicolor* whiB2 plasmid was generated by PCR amplifying the whiB2 gene, including its promoter sequence, from *S. coelicolor* A3(2) genomic DNA using the primers pBP10whmD1-2 (5′ AAAATTGACGAATCCTGCGC CTGAGAC 3′) and pBP10whmD1-2 (5′ GGAATTCTTGAATCTGCGC CTGACG 3′) and cloned at the PstI-SpeI sites of pBP10 zeo to generate pBP10 zeo whiB2. All clones generated as described above were confirmed by sequencing. **Construction of mutant alleles of WhmD.** All deletion mutants of WhmD were constructed using a PCR-based strategy. To generate the N-terminus deletion mutant, the *whmD* promoter fragment, amplified using the PCR primers pBP10whmD-F (5′ AAAATTGACGAATCCTGCGC CTGAGAC 3′) and pBP10whmD-R (5′ GGAATTCTTGAATCTGCGC CTGACG 3′), was fused to a fragment of *whmD* carrying the appropriate deletion and cloned at the PstI-SpeI sites of pBP10 zeo. The fusion, mediated by a BamHI site, introduces a Gly-Ser dipeptide at the junction of the Met encoded by the start codon and the WhmD fragment carrying the N-terminal deletion. The addition of this dipeptide should interfere with WhmD protein function in the complementation assay.

**Construction of deletion mutants using PCR amplifying *whmD* by using the forward primer pBP10whmD-F (5′ AAAACTGGAATCCTGCGC CTGAGAC 3′) and the reverse primers used to amplify *whmD* depending on the required length of the deletion, following which were cloned at the PstI-SpeI sites of pBP10 zeo. The reverse primers used to generate the C-terminal deletions were as follows: whmD/C2-13 (5′ GCGATACCTGACGACGCGG GCTGACGACGCGG 3′) and whmD/C2-24 (5′ GCGATACCTGACGACGCGG GCTGACGACGCGG 3′); and whmD/C2-9 (5′ GCGATACCTGACGACGCGG GCTGACGACGCGG 3′) and whmD/C2-26 (5′ GCGATACCTGACGACGCGG GCTGACGACGCGG 3′). All site-directed mutants were generated using the Quick-Change mutagenesis strategy (Stratagene) in the plasmid pBP10 zeo with *Pfu* DNA polymerase and primer pairs carrying the desired mutation. The following primer pairs were used: C67A-1 (5′ GCGAGGATGCGGCTTGGCCG GCCGCACTCGGACC 3′) and C67A-2 (5′ CCGGCTTTTAATGGCCTGGC 3′); C90A-1 (5′ GCCGAACTGGGCGCCGGCGGGCCGG 3′) and C90A-2 (5′ CCGGCTTTTAATGGCCTGGC 3′); C122A-1 (5′ ATGCTGTCGCTCAGAAGCTCCCGAGCGGCGG CCGGCACTCGGACC 3′) and C122A-2 (5′ ATGCTGTCGCTCAGAAGCTCCCGAGCGGCGG CCGGCACTCGGACC 3′).
carbolfuchsin for 5 min. After the excess dye was washed off with distilled water, the slides were dried and examined at a ×600 or ×1,000 magnification on a Nikon Eclipse E800 microscope under oil. Images were captured using an onboard digital still camera (model DXM1200) and edited using the software package ACT-1 version 2. Cell length measurements were made to determine the extent of complementation of each allele. Transformants with a mean cell length of ≥7.5 μm were scored as complemented.

**Western blotting.** To quantitate protein levels of the point mutants and C-terminal deletion mutants of WhmD, transformants of *M. smegmatis* 628-53 containing each complementing allele and the controls pBP10zeo and pBP10zeo whmD were grown to an optical density of 1.0 in the presence of 0.2% acetamide, washed twice with 7H9, resuspended in 7H9 broth, and grown overnight (~16 h) in the absence of acetamide. Following inducer withdrawal, cultures were washed and resuspended in phosphate-buffered saline and lysed by bead beating on a mini bead beater (Biospec Products, Bartlesville, OK) and the amount of protein was quantitated by the Bradford assay (3). Thirty micrograms of total cell lysate proteins and 3 μg of purified WhmD (rWhmD) were electrophoresed on a 12% SDS-PAGE system and transferred to nitrocellulose. For Western blotting, WhmD antiserum was used at a 1:200 dilution. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G at a 1:3,500 dilution and a chemiluminescent substrate (Amer sham) were used to detect the presence of WhmD and its mutant alleles.

**Purification of *M. smegmatis* WhmD.** The gene encoding WhmD was PCR amplified from *M. smegmatis* chromosomal DNA using the gene-specific primers pET22bwhmD-F (5’-GGGAATTCATATGTCTTATGAGAGCGGC-3’) and
pET22bwhmD-R (5′-CCGCTCGAGGATGATGCCGCGCTTGAGG3′). The amplified product was cloned into the expression vector pET-22b(+) at the NdeI and XhoI sites, and the recombinant plasmid was used to transform E. coli BL21(DE3). The transformant culture was grown to exponential phase, induced with 1 mM isopropyl-thio-D-galactoside (IPTG) for 2 h at room temperature, and lysed by sonication. C-terminal hexahistidine-tagged WhmD was purified from the soluble fraction by Ni-nitrilotriacetic acid chromatography according to the manufacturer’s protocol (QIAGEN, Valencia, CA). The purity of the protein was analyzed by SDS-PAGE. To overexpress the cysteine-to-alanine point mutants of WhmD, the mutant open reading frames (ORFs) were amplified from their cognate mutant constructs in pBP10 zeo whmD using the primers pET22whmD-F and pET22bwhmD-R and cloned into pET-22b(-). The expression and purification regimens were as described above. Native PAGE and SDS-PAGE was performed as described previously (20). The 4× SDS-PAGE sample buffer used either contained or lacked 5 mM β-mercaptoethanol. 

Iron staining. Staining was carried out as described by Kuo and Fridovich (15). All procedures were carried out at room temperature. Briefly, 9 μg of purified WhmD was electrophoresed on a 10% native PAGE system and immersed in a solution of 50 mM sodium acetate, pH 5.0. H2O2 from a 30% (8 M) stock solution was added to 40 mM, and dianisobenzoxoic acid dihydroxide from a freshly prepared 0.8 M solution was added to a final concentration of 80 mM. The gel was gently agitated for 30 min, following which the staining solution was decanted, twice rinsed with water, and then placed in 7% acetic acid. The gel was photographed following the appearance of visible staining.

Sequence analysis. All sequence alignments were performed with the BCM search launcher in the Multiple Sequence Alignment package (Baylor College of Medicine) using the ClustalW 1.8 algorithm. The output files were imported into Boxshade 3.21 (www.ch.embnet.org) to generate the formatted alignments shown in Fig. 2. The secondary-structure prediction for WhmD was carried out with the NPS@ package on the Pole BioInformatique Lyonnais server (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html). The boundary coordinates of the 5′ UTR sequences shown in the alignment in Fig. 2B, with reference to their locations upstream of the start codon, are as follows: for M. smegmatis whmD, −187 to −70; for M. tuberculosis whiB2, −185 to −68; and for S. coelicolor whiB, −114 to −13.

RESULTS

A wild-type copy of M. smegmatis whmD rescues the conditionally complemented whmD mutant M. smegmatis 628-53, providing a genetic assay for screening nonfunctional mutants of WhmD. M. smegmatis 628-53, the conditionally complemented whmD deletion mutant, undergoes filamentation under nonpermissive conditions (10). In order to establish a genetic assay to identify mutations that disrupt WhmD function, we developed a complementation system based on M. smegmatis 628-53. To determine if a wild-type (WT) copy of whmD rescues the filamentation defect of the mutant, the gene was cloned under the control of its own promoter into the vector pBP10. This vector exists as a single-copy episome in mycobacteria (2). This was critical since M. smegmatis 628-53 contains pJG1012, a pAL5000-based plasmid expressing whmD under the control of the acetylomamide promoter (Pswm). To facilitate selection, a gene encoding Zeocin (zeo) resistance was inserted into pBP10. As depicted schematically in Fig. 1A, transformants of M. smegmatis 628-53 containing either pBP10 zeo whmD or pBP10 zeo alone were cultured in acetamide-containing medium and then subjected to inducer withdrawal as described in the experimental procedures. Following acetamide withdrawal, cells were stained with carbol-fuchsin and visualized by light microscopy. Transformants containing the control plasmid were highly filamentous (Fig. 1B, left), whereas no filamentation was seen in those containing the extra copy of whmD (Fig. 1B, right). Cell length measurements were made to quantitate the extent of complementation. The appreciable differences in cell length in control versus WT whmD-complemented cells (Table 1) provided us with a robust system for examining complementation phenotypes of various alleles of whmD.

Orthologues of M. smegmatis whmD rescue M. smegmatis 628-53. The homologues of M. smegmatis whmD in M. tuberculosis and S. coelicolor show extensive identity at the protein level. WhmD shares 70% identity with M. tuberculosis WhiB2 and 69% identity with S. coelicolor WhiB (Fig. 2A). In addition, the three genes also share a high degree of homology in their 5′ and 3′ UTRs (data not shown). To examine if the two genes are truly orthologous to M. smegmatis whmD, the S. coelicolor whiB and M. tuberculosis whiB2 ORFs, including 200 bp of their 5′ UTRs, were cloned into pBP10 zeo and put through the complementation assay described above. As shown in Fig. 2C and Table 1, both S. coelicolor WhiB and M. tuberculosis WhiB2 functionally complement M. smegmatis 628-53, as assessed by rescue of filamentation, implying that these genes are true orthologues of M. smegmatis whmD. The fact that complementation was observed also indicates that the promoter sequences driving the expression of S. coelicolor whiB and M. tuberculosis whiB2 are functional in M. smegmatis. A sequence alignment of the 5′ UTRs of the three genes shows that the putative promoter elements are highly conserved (Fig. 2B).

<table>
<thead>
<tr>
<th>Vector, complementing allele, or</th>
<th>Cell length (%)</th>
<th>% of cells below cutoffa</th>
</tr>
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<tr>
<td>WhmD mutation</td>
<td>WhmD 6969</td>
<td>628-53</td>
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<tr>
<td>vector (pBP10 zeo)</td>
<td>23.28 ± 2.2</td>
<td>0</td>
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<tr>
<td>M. smegmatis WhmD</td>
<td>5.16 ± 0.31</td>
<td>100</td>
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<tr>
<td>S. coelicolor WhiB</td>
<td>7.33 ± 0.48</td>
<td>65</td>
</tr>
<tr>
<td>M. tuberculosis WhiB2</td>
<td>6.95 ± 0.5</td>
<td>80</td>
</tr>
</tbody>
</table>

Table 1. Cell length measurements of transformants of M. smegmatis 628-53 containing the complementing alleles generated in this study.

Values represent means ± standard errors of the means (n = 30). ND, not determined.

Percentage of cells below the cell length cutoff of ≤7.5 μm, which defines a filament.
The N-terminal extension of WhmD is not essential for its activity. Sequence alignment of WhmD and its orthologues (Fig. 2A) shows that WhmD and WhiB2 carry N-terminal extensions missing in S. coelicolor WhiB and M. leprae WhiB2 (data not shown). To clarify if the N-terminal extension contributes significantly to WhmD function, we generated sequential N-terminal deletions in WhmD in pBP10 using a PCR-based strategy. Three of the deletion mutants created (WhmD ΔN42, WhmD ΔN57, and WhmD ΔN62) carried nested deletions of the first three predicted helices. The largest deletion (ΔN67) lacks the first 67 amino acids, including the first of the four conserved cysteine residues, C67 (Fig. 3). All these mutants were tested for their ability to rescue the filamentation phenotype of M. smegmatis 628-53. None of the four mutants were compromised in their ability to complement the mutant (Fig. 4A; Table 1), suggesting that the N-terminal half of WhmD is dispensable for activity, at least under the conditions tested. It was surprising that deletion of the first

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**Figure 2.** Orthologues of *M. smegmatis* *whmD* rescue *M. smegmatis* 628-53. (A) Protein sequence alignments of *M. smegmatis* WhmD (MsWhmD), *S. coelicolor* WhiB (ScWhiB), and *M. tuberculosis* WhiB2 (MtWhiB2). (B) Alignments of the 5’ UTRs of *M. smegmatis* *whmD*, *S. coelicolor* *whiB*, and *M. tuberculosis* *whiB2*, showing the positions of the promoter elements. The four-pointed star marks the transcription start site as determined by 5’ rapid amplification of cDNA ends. (C) Morphology upon inducer withdrawal of *M. smegmatis* 628-53 transformed with plasmids containing *S. coelicolor* *whiB* (1) and *M. tuberculosis* *whiB2* (2).
conserved cysteine had no effect on WhmD function, an observation which was later confirmed by site-directed mutagenesis.

Truncations in the putative HTH motif of WhmD are deleterious to WhmD function. The C terminus of WhmD is predicted to contain a putative HTH-like domain, believed to be involved in DNA binding (21). As shown in Fig. 3, this domain is likely to be comprised of predicted helices 5 and 6, interspersed with a β-turn. To investigate the significance of this domain, we constructed three C-terminal WhmD truncations which progressively deleted portions of this domain and tested them in the complementation assay described above. We observed a progressive debilitation in protein function as deletions in the C terminus (and as a consequence, into the HTH) got larger. The mutant lacking 12 amino acids from the C terminus, which deletes predicted helix 6, retained activity (Fig. 4B, top left, and Table 1), whereas the ΔC23 mutant, which carries a deletion in the predicted turn as well, showed a partial complementation phenotype (Fig. 4B, top right, and Table 1). ΔC30, the largest deletion, which removed part of helix 5 as well as the turn and helix 6, failed to complement the mutant (Fig. 4B, bottom, and Table 1). These observations together suggest that an intact HTH motif is required for the optimal functioning of WhmD. To ensure that the lack of complementation of the deletion mutants tested was not due to the instability of the mutant proteins, we probed cell lysates of those alleles (and as a consequence, into the HTH) for their instability in vivo (Fig. 4B, top right, and Table 1). ΔC30, the largest deletion, which removed part of helix 5 as well as the turn and helix 6, failed to complement the mutant (Fig. 4B, bottom, and Table 1). These observations together suggest that an intact HTH motif is required for the optimal functioning of WhmD. To ensure that the lack of complementation of the deletion mutants tested was not due to the instability of the mutant proteins, we probed cell lysates of those alleles (and as a consequence, into the HTH) for their instability in vivo (Fig. 4B, top right, and Table 1). ΔC30, the largest deletion, which removed part of helix 5 as well as the turn and helix 6, failed to complement the mutant (Fig. 4B, bottom, and Table 1). These observations together suggest that an intact HTH motif is required for the optimal functioning of WhmD. To ensure that the lack of complementation of the deletion mutants tested was not due to the instability of the mutant proteins, we probed cell lysates of those alleles (and as a consequence, into the HTH) for their instability in vivo.
gests that the WhiB-like proteins may be sensitive to redox changes, perhaps through a bound metal atom or through direct sensitivity to oxidation via disulfide bond formation. To determine if either of these possibilities is likely, the whmD ORF was cloned and expressed in the vector pET22b(H11001) with a C-terminal hexahistidine tag. Following purification from the soluble fraction using conventional metal affinity chromatography, WhmD was electrophoresed on a denaturing SDS-PAGE system. Curiously, a difference in protein mobility was observed between samples lacking or containing the reducing agent β-mercaptoethanol (Fig. 7A, left, lane 1 versus lane 2). The same difference was observed when WhmD was expressed with an N-terminal glutathione S-transferase fusion, indicating that the phenomenon was not an artifact of the expression system (data not shown). We hypothesized that perhaps the reducing agent disrupted the coordination between the cysteines.
tein residues and a metal ion, leading to a loss of compaction in protein conformation and a reduction in mobility (Fig. 7B). On treatment of the protein with 1 mM EDTA followed by SDS-PAGE, both forms of the protein were observed (data not shown), consistent with the presence of a bound metal. No difference in mobility was observed when WhmD C67A was electrophoresed under identical conditions (Fig. 7A, right), a result implicit in the fact that the cysteine residues coordinate a metal ion. The same was observed for the three other cysteine mutations, C90A, C93A, and C99A (data not shown).

FIG. 5. Estimation of the stability of complementing mutant alleles of WhmD. Western blot analysis of complementing C-terminal deletion mutant alleles (A) and point mutant alleles (B) of WhmD following inducer withdrawal. M, molecular mass marker; rWhmD, purified recombinant WhmD.

FIG. 6. Phenotypic consequences of mutagenizing conserved residues in WhmD. (A) Complementation phenotypes of the four cysteine-to-alanine mutants of WhmD. (B) The inactivation of a conserved aspartate (left) and a glycine (middle) inactivate WhmD. The L116P mutation mimics the inactive S. coelicolor whiB70 allele (right).
Iron-specific staining (15) of WT WhmD electrophoresed on a 10% native PAGE system (Fig. 7C) strongly suggested that the metal ion coordinated by the cysteine residues was iron. Interestingly, the native gel electrophoretic profile indicated that WhmD forms oligomers and iron staining was observed to be uniform over the entire profile.

**DISCUSSION**

The WhiB-like proteins have been associated with a myriad of functions, including sporulation in *S. coelicolor* (5, 8), septum formation in *M. smegmatis* (10), pathogenesis in *Mycobacterium marinum* (19), transcription in *M. tuberculosis* (22), antibiotic resistance in the mycobacteria and streptomycetes (16), and survival following oxidative stress in *C. glutamicum* (14). Despite advances in our understanding of the physiological roles of the WhiB-like proteins in the actinomycetes, their structure-function relationships as well as the significance of the conserved set of residues in all these proteins are poorly understood. To address the above issues, we set out to map the essential regions and residues of WhmD and the WhiB-like protein, essential for septation in *M. smegmatis*. A single-copy complementation assay was developed for *M. smegmatis* 628-53 to allow screening for nonfunctional mutants of WhmD.

*M. tuberculosis* whiB2 and *S. coelicolor* whiB were found to complement the *M. smegmatis* whmD mutant. The three proteins shared extensive identity towards their C termini, so this observation was no surprise. In addition, since the two homologous genes were cloned into the complementation vector under the control of their own promoters, this indicated that the two heterologous promoter sequences were active in *M. smegmatis*. Alignment of the three 5′ UTR sequences suggested that this was likely to be due to the near identity of the predicted −10 and −35 elements. Transcription start site mapping confirmed that, for *M. smegmatis* whmD and *M. tuberculosis* whiB2, the actual promoter elements are the predicted hexamers shown here (our unpublished data). The complementation analysis allowed us to conclude that *S. coelicolor* whiB and *M. tuberculosis* whiB2 are truly orthologous to *M. smegmatis* whmD. The corollary of this observation is that the results obtained from the functional analysis of WhmD can in principle be extended to the two orthologous proteins as well.

Large deletions in the N-terminal region of WhmD seemed not to compromise protein function, implying that the ex-
tended N terminus is apparently superfluous. It is conceivable that this observation is specific to the conditions under which the complementation was performed, and the extension might be required in an independent assay system. It is also possible that WhmD plays other roles beyond regulating septation and that the N terminus is important for one of these nonseptation functions. The result from the largest deletion, which included C67, a conserved residue, was partly unexpected, since we anticipated that all four cysteines would be essential for activity. This observation was initially thought to be an artifact but was later confirmed using a site-directed mutagenesis approach, where three of the four cysteines were found to be indispensable, and WhmD C67A retained its ability to complement M. smegmatis 628-53. In S. coelicolor WhiD, the four cysteine residues are believed to be involved in binding a [4Fe-4S] cluster (13). The functional importance of this cluster was emphasized by the observation that none of the four whiD alleles carrying mutations at these cysteine residues was able to complement the whiD mutant phenotype in S. coelicolor. In this study, we observed that the mobility of purified WhmD changed in response to a reducing agent, an effect not seen in all four mutants of WhmD with cysteine-to-alanine mutations. In addition, WhmD also displayed iron-specific staining, strongly suggesting that in all probability the protein coordinates an iron-sulfur cluster. In light of the nonessentiality of C67, it was interesting that WhmD D71A was inactive, implying that this aspartate residue is functionally essential. It is conceivable later confirmed using a site-directed mutagenesis approach, that this observation is specific to the conditions under which the complementation was performed, and the extension might be required in an independent assay system. It is also possible that WhmD plays other roles beyond regulating septation and that the N terminus is important for one of these nonseptation functions. The result from the largest deletion, which included C67, a conserved residue, was partly unexpected, since we anticipated that all four cysteines would be essential for activity. This observation was initially thought to be an artifact but was later confirmed using a site-directed mutagenesis approach, where three of the four cysteines were found to be indispensable, and WhmD C67A retained its ability to complement M. smegmatis 628-53. In S. coelicolor WhiD, the four cysteine residues are believed to be involved in binding a [4Fe-4S] cluster (13). The functional importance of this cluster was emphasized by the observation that none of the four whiD alleles carrying mutations at these cysteine residues was able to complement the whiD mutant phenotype in S. coelicolor. In this study, we observed that the mobility of purified WhmD changed in response to a reducing agent, an effect not seen in all four mutants of WhmD with cysteine-to-alanine mutations. In addition, WhmD also displayed iron-specific staining, strongly suggesting that in all probability the protein coordinates an iron-sulfur cluster. In light of the nonessentiality of C67, it was interesting that WhmD D71A was inactive, implying that this aspartate residue is functionally essential. It is conceivable that in the absence of C67, D71 could play a role in binding the cluster along with C90, C93, and C99, as seen in ferredoxin III from Desulfovibrio africanus (9). It is also intriguing that, of the 121 bacterial WhiB-like proteins in the Pfam database, the only protein which shows a deviation from the four-cysteine conservation (Tropheryma whipplei TW 636, Pfam entry Q83NC4) is missing the first cysteine residue. We initially hypothesized, based on the conservation of the aspartate residue, that WhmD could be part of a two-component sensorresponse regulator system (6) and that D71 might serve as a phosphor acceptor. This possibility is unlikely since such systems are usually modular (23), and WhmD possesses no sequence resembling the consensus CheY-like phosphorylation site seen in most two-component response regulators (25). Indeed, the present study suggests that WhmD Asp71, while essential, might be required in order to coordinate a metal ion rather than serve as a phosphor acceptor. Structural evidence strongly suggests that the WhiB-like proteins are DNA binding proteins. The four-conserved-cysteine signature is a common motif in metal-coordinating DNA binding proteins such as Zn-binding GAL4, Fe-binding SoxR, or Hg-binding MerR (18). Secondary-structure analysis of S. coelicolor WhiB showed a potential DNA binding HTH-like motif from residues 64 to 84, and the importance of this motif was emphasized by the loss of WhiB activity in the whiB70 allele in which Leu74 is replaced by a proline residue. In M. smegmatis WhmD, the predicted HTH lies between residues 93 and 126, and the cognate L116P mutation in WhmD led to partial inactivation of protein function, underscoring the importance of this motif. Additional support for the significance of this motif was provided by the observations that a deletion into helix 5 and a mutation in the turn (G111P) were not tolerated. We were puzzled by the retention of activities of the mutants carrying helix-disrupting substitutions in helix 5 (Y102G, Y102P) and helix 6 (R122G, R122P), since these helices are integral parts of the HTH motif. A simplistic explanation is that the protein retains its functional conformation despite the kinks generated by inserting proline residues in the putative helices, as seen in the Saccharomyces cerevisiae heat shock transcription factor (11). The nondeleterious effect of ΔC12 on WhmD was surprising as well, since the deletion removes the terminal helix. If WhmD functions by directly regulating the expression of genes involved in septum formation, then in principle, this deletion mutant should have been unable to rescue filamentation in M. smegmatis 628-53. Since WhmD has the propensity to oligomerize (Fig. 7C), it is plausible that this C-terminal deletion mutant could form heterooligomers with other WhiB-like proteins and compensate for the DNA binding defect. All the above issues can be unequivocally resolved by examining the biochemical properties of the mutants constructed in this study. From the above analyses, we have delineated the essential region of WhmD as lying between residues 68 and 106. This region includes 6 of the 10 residues conserved in all seven M. tuberculosis WhiBs as well as in most members of the WhiB family identified to date. To the best of our knowledge, this is the first comprehensive report documenting the structurefunction relationships in the WhiB-like protein family.

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