JOURNAL OF BACTERIOLOGY, Oct. 2006, p. 6966–6976 Vol. 188, No. 19
0021-9193/06/$08.00+0 doi:10.1128/JB.00384-06

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

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Received 17 March 2006/Accepted 19 June 2006

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 whiB2* 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 described to map to eight independent loci (5, 12). The first-characterized nonsporulating mutant was shown to be defective in *whiB*, a gene encoding a basic polypeptide 87 amino acids long with a putative helix-turn-helix (HTH) motif (8). *whiB* mutants are viable but incapable of sporulating. Secondary-structure prediction and mutational analysis suggest that WhiB is a DNA binding protein (8). In addition, *whiB* mutants show reduced expression of the *whiE* cluster encoding spore pigment, indicating that this gene is likely to be a transcription factor.

The large database of DNA sequences of members of the order *Actinomycetales* has revealed a family of genes encoding proteins related to WhiB. The list is currently comprised of 121 bacterial proteins (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF02467) exclusive to the high-GC-content actinomycete genera, including *Streptomyces*, *Rhodococcus*, and *Mycobacterium*. Each of these proteins contains four invariant cysteine residues arranged as Cys-$X_{14–22}$-Cys-X_2-Cys-X_5-Cys and a conserved HTH-like motif characterized by the 7-residue signature (FYG)-G-(VI)-W-G-G-(LVIM) in the putative β-turn and is relatively short in length (76 to 139 residues). Although this motif does not comprise a typical HTH motif (1), it is believed 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 (*whiB1* to *whiB7*) (7, 21) 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.

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Although the most C-terminal α-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 WhiB-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 irreversible 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 composed largely of helical and coiled regions with two probable sheet regions, the second of the two being a part of the putative HTH-like motif. Using WhmD as a prototype, we have examined the role of the conserved residues as well as the predicted structural modules in WhiB function. Using a combination of deletion and site-specific mutation analysis followed by genetic complementation, we have mapped regions and residues essential to the function of WhmD in *M. smegmatis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* strain DH5α {F′ *endA1 hsdR17*(r_K^- m_K^+) *glnV44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169 deoR* [ϕ80*dlac*Δ(*lacZ*)*M15*]} procured from Stratagene, La Jolla, CA, was used for cloning purposes. *E. coli* BL21(DE3) [F^- *ompT* *hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)] used for protein expression was purchased from Novagen, Madison, WI. *M. smegmatis* mc^26 1-2c was kindly provided by Bill Jacobs, Albert Einstein College of Medicine, New York, NY, and *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 *E. coli* and 50 μg/ml for mycobacteria).

DNA techniques. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (NEB), Beverly, MA, and *Taq* polymerase was purchased from Invitrogen Corporation, CA. *Pfu* DNA polymerase was purchased from Stratagene, CA. The Klenow fragment of DNA polymerase was from NEB. Protocols for DNA manipulations, including plasmid DNA preparation, restriction endonuclease digestion, agarose gel electrophoresis, isolation and ligation of DNA fragments, *E. coli* transformation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were performed as described by Sambrook et al. (20). Mycobacterial strains were transformed by electroporation. PCR amplifications were carried out according to the manufacturer's specifications (Bio-Rad Laboratories, CA). Each of the 30 cycles was carried out at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension cycle at 72°C for 10 min. DNA fragments used for cloning and for labeling reaction products were purified by using the QIAGEN gel extraction kit (QIAGEN) per the manufacturer's specifications.

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. *whmD* containing 187 bp of its 5′ untranslated region (UTR) was amplified from *M. smegmatis* genomic DNA using the PCR primers pBP10*whmD*-F (5′ AAAACTGCAGGAATTCGCGCC CTGGAGC 3′) and pBP10*whmD*-R (5′ GGACTAGTCTAGATGATGCCGC GCTT 3′) and cloned at the PstI-SpeI sites of pBP10 *zeo* to generate pBP10 *zeo whmD*. The *S. coelicolor whiB* test plasmid was generated by PCR amplifying the *whiB* gene, including its promoter sequence, from *S. coelicolor* A(3)2 genomic DNA using the primers pBP10*ScwhiB*-F (5′ AAAACTGCAGGACTTCATTG GTATGTCG 3′) and pBP10*ScwhiB*-R (5′ GGACTAGTCTGCACGGACATC GAGGT 3′), followed by cloning into the PstI and SpeI sites of pBP10 *zeo*. *whiB2* was amplified from *M. tuberculosis* CDC1551 genomic DNA by using the PCR primers pBP10*MtwhiB2*-F (5′AAAACTGCAGTTACGAGATGATATGGAA 3′) and pBP10*MtwhiB2*-R (5′ GGACTAGTTCAGATGATCCCGCGTTT 3′) and cloned into the PstI and 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-terminal deletion mutants, the *whmD* promoter fragment, amplified using the PCR primers pBP10 *whmD*-F (5′ AAAACTGCAGGAATTCGCGMLCTGGAGC 3′) and P*whmD*Met-R (5′ CGCGGATCCCATATCMLCCGCCTCCTC 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 does not interfere with WhmD protein function in the complementation assay. The following forward primers, in conjunction with the *whmD* reverse primer pBP10*whmD*-R (5′ GGACTAGTCTAGATGATGCCGCGCTT 3′), were used to generate the following N-terminal deletions: *whmD*ΔN42-F (5′ CGCGGATCCCTGAGTCTGGTGCCCGAT 3′), *whmD*ΔN57-F (5′ CGCGG ATCCGAAGACCAATGGCAGGAG 3′), *whmD*ΔN62-F (5′ CGCGGATCCG AGCGTGCCCTGTGCGCG 3′), and *whmD*ΔN67-F (5′ CGCGGATCCGCGC AAACTGACCCGGAG 3′). C-terminal deletion mutants were generated by PCR amplifying *whmD* using the forward primer pBP10*whmD*-F (5′ AAAACT GCAGGAATTCGCGCCCTGGAGC 3′) and a combination of reverse primers depending on the required length of the deletion, following which the products were cloned at the PstI-SpeI sites of pBP10 *zeo*. The reverse primers used to generate the C-terminal deletions were as follows: *whmD*ΔC12-R (5′GGACTA GTCTACGACAGACCGCCCCA 3′), *whmD*ΔC23-R (5′ GGACTAGTCTAAT GCGCGAGCGCGTA 3′), and *whmD*ΔC30-R (5′ GGACTAGTCTAGCACG CGTCACGAAC 3′). All site-directed mutants were generated using the QuikChange mutagenesis strategy (Stratagene) in the plasmid pBP10 *zeo whmD* with *Pfu* DNA polymerase and primer pairs carrying the desired mutation. The following primer pairs were used: C67A-1 (5′ GCAGGAGCGTGCCCTGGCC GCGCAAACTGACCCG 3′) and C67A-2 (3′ CGTCCTCGCACGGGACCGG CGCGTTTGACTGGGC 5′); C90A-1 (5′ GAGGCCAAGCGCATCGCCCAG GGGTGCGAAGTTCG 3′) and C90A-2 (3′ CTCCGGTTCGCGTAGCGGGT CCCCACGCTTCAAGC 5′); C93A-1 (5′ CGCATCTGCCAGGGGGCCGAA GTTCGTGACGCG 3′) and C93A-2 (3′ GCGTAGACGGTCCCCCGGCTTC AAGCACTGCGC 5′); C99A-1 (5′ CGAAGTTCGTGACGCGGCCCTGGAA TACGCGCTCG 3′) and C99A-2 (3′ GCTCGCGCATAAGGTCCGGGCGCA GTGCTTGAAGC 5′); Y102G-1 (5′ GACGCGTGCCTGGAAGGCGCGCTC GCGCATGATG 3′) and Y102G-2 (3′ CTGCGCACGGACCTTCCGCGCGA GCGCGTACTAC 5′); Y102P-1 (5′ GACGCGTGCCTGGAACCGGCGCTCG CGCATGATG 3′) and Y102P-2 (3′ CTGCGCACGGACCTTGGCCGCGAG CGCGTACTAC 5′); G111P-1 (5′ GCATGATGAGCGCTTCCCGATCTGGG GCGGTCTG 3′) and G111P-2 (3′ CGTACTACTCGCGAAGGGCTAGACC CCGCCAGAC 5′); R122G-1 (5′ CTGTCGGAGCGTGAGCGCGGCCGCCT CAAGCGCGGC 3′) and R122G-2 (3′ GACAGCCTCGCACTCGCGGGCGC GGAGTTCGCGCCG 5′); and R122P-1 (5′ CTGTCGGAGCGTGAGCGCCC GCGCCTCAAGCGCGGC 3′) and R122P-2 (3′ GACAGCCTCGCACTCGC GGGCGCGGAGTTCGCGCCG 5′). All deletion and site-directed *whmD* mutants were confirmed by sequencing using the primer *whmD*seq (5′ MLTCA ACTGAGTCTGGTGCC 3′).

***M. smegmatis* 628-53 complementation assays.** For complementation analysis, the test allele and the control plasmid pBP10 *zeo* were transformed into *M. smegmatis* 628-53 and selected on 0.2% acetamide-supplemented 7H10 agar plates containing apramycin, hygromycin, and Zeocin. Acetamide withdrawal was performed as follows: transformants were cultured in 7H9 broth supplemented with 0.2% acetamide, grown to an optical density of 1.0, washed twice with 7H9 broth, 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, heat fixed on slides, and stained with

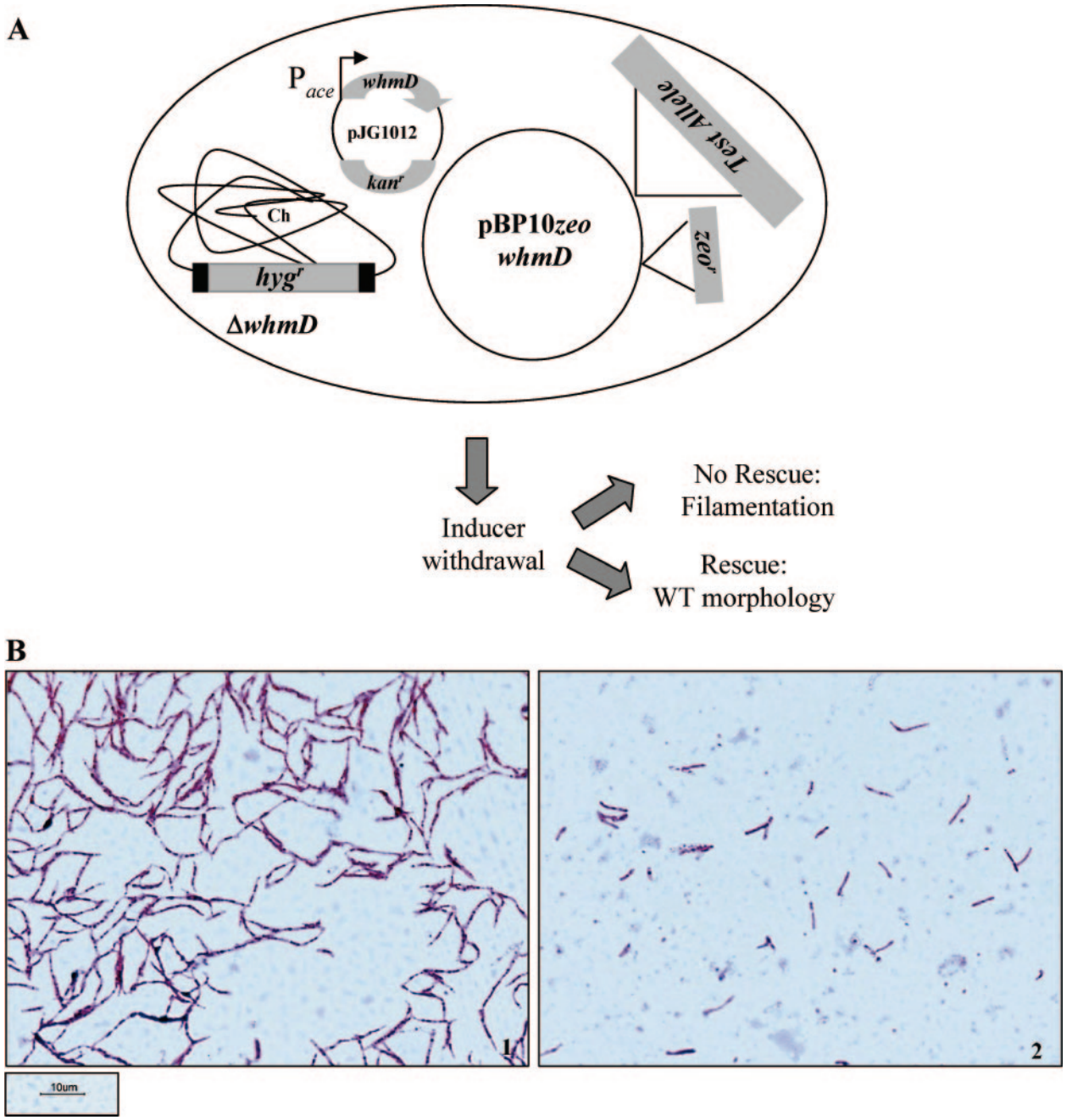


FIG. 1. A wild-type copy of *M. smegmatis whmD* rescues the conditionally complemented *whmD* mutant *M. smegmatis* 628-53. (A) Schematic of assay system to screen nonfunctional mutants of WhmD. Ch, chromosome. (B) Morphology upon inducer withdrawal of vector-transformed (1) and *whmD*-transformed (2) *M. smegmatis* 628-53.

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 on-board 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 pBP10 *zeo* and pBP10 *zeo 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 (Amersham) 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 pET22b*whmD*-F (5′ GGGAATTCCATATGTCTTATGAGAGCGGC 3′) and

pET22b*whmD*-R (5′ CCGCTCGAGGATGATGCCGCGCTTGAG 3′). 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-β-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 pET22b*whmD*-F and pET22b*whmD*-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. H_2O_2 from a 30% (8 M) stock solution was added to 40 mM, and diaminobenzoic acid dihydrochloride 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://npsa-pbil.ibcp.fr) and the PSIPRED protein structure prediction 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.

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

Vector, complementing allele, or WhmD mutation	Cell length (μm)[a]	% of cells below cutoff[b]
Vector (pBP10 *zeo*)	23.28 ± 2.2	0
M. smegmatis WhmD	5.16 ± 0.31	100
S. coelicolor WhiB	7.33 ± 0.48	65
M. tuberculosis WhiB2	6.95 ± 0.5	80
M. smegmatis WhmD mutations		
ΔN42	4.55 ± 0.39	90
ΔN57	5.35 ± 0.46	90
ΔN62	4.39 ± 0.53	90
ΔN67	7.12 ± 0.44	65
ΔC12	6.14 ± 0.33	76.6
ΔC23	10.51 ± 0.6	20
ΔC30	22.6 ± 1.59	0
C67A	6.43 ± 0.4	63
C90A	ND	
C93A	20.1 ± 1.73	0
C99A	19.44 ± 2.49	0
D71A	24.29 ± 2.29	3.33
Y102G	6.95 ± 0.63	66.6
Y102P	5.66 ± 0.38	83.3
G111P	25.7 ± 1.65	0
L116P	13.2 ± 1.18	16.6
R122G	6.12 ± 0.54	83.3
R122P	5.23 ± 0.22	93.3

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

[b] Percentage of cells below the cell length cutoff of ≤7.5 μm, which defines a filament.

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 mycobacterial cells and contains an origin of replication compatible with the pAL5000 replicon (2). This was critical since *M. smegmatis* 628-53 contains pJG1012, a pAL5000-based plasmid expressing *whmD* under the control of the acetamidase promoter (P_{ace}). 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 carbolfuchsin 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).

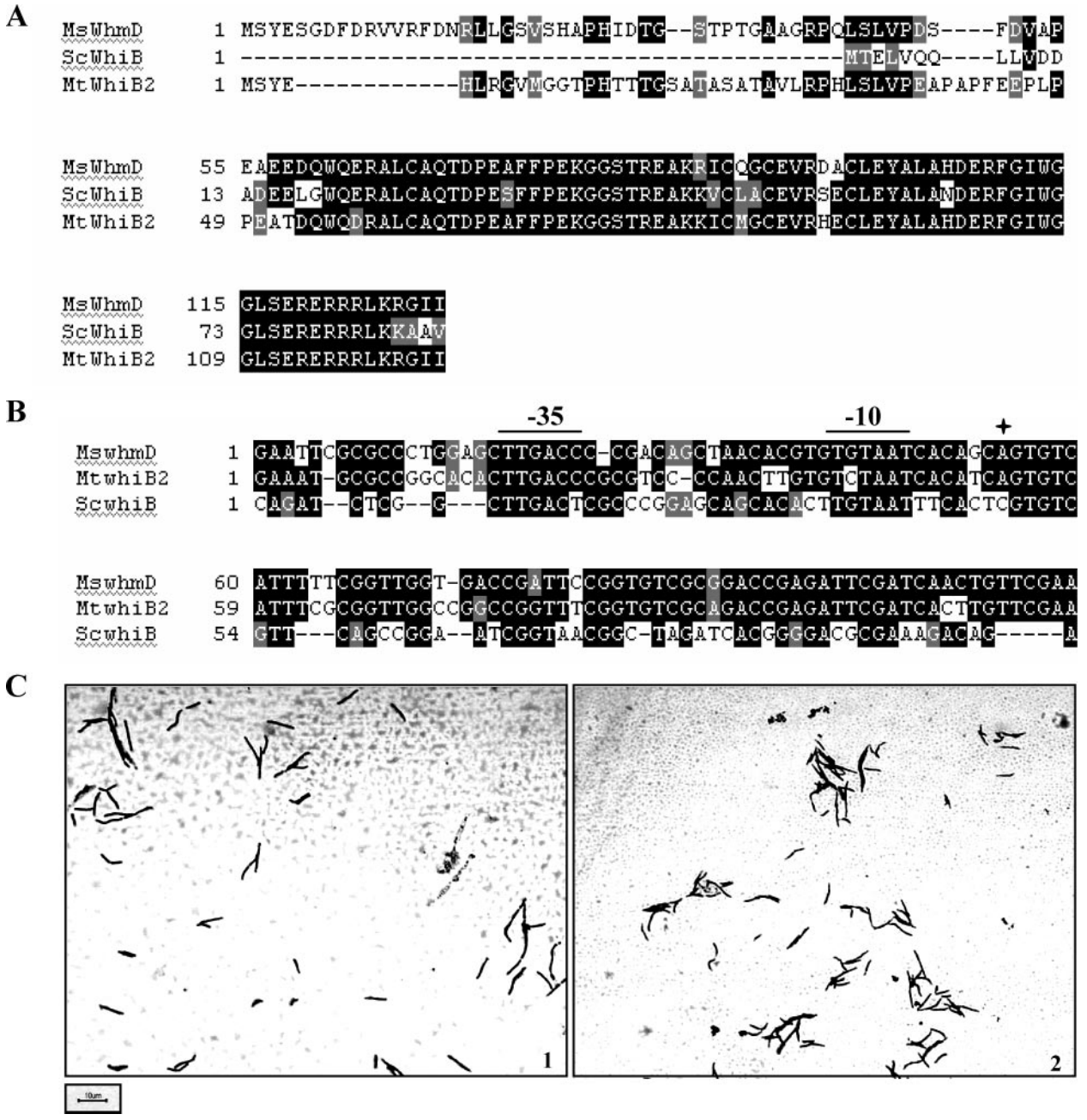
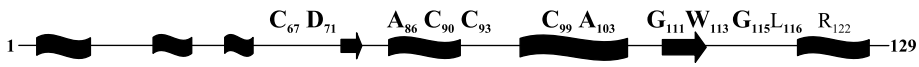


FIG. 2. Orthologues of *M. smegmatis whmD* rescue *M. smegmatis* 628-53. (A) Protein sequence alignments of *M. smegmatis* WhmD (MsWhmD), *S. coelicolor* WhiB (ScWhiB), and *M. tuberculosis* WhiB2 (MtWhiB2). (B) Alignments of the 5′ UTRs of *M. smegmatis whmD*, *S. coelicolor whiB*, and *M. tuberculosis 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).

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 *Mycobacterium 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, C_{67} (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



Feature	Helix 1	Helix 2	Helix 3	Strand 1	Helix 4	Helix 5	Strand 2	Helix 6
aa	8-22	44-51	59-62	74-75	83-91	96-105	111-113	118-126

FIG. 3. Schematic representation of WhmD showing the coordinates of the predicted secondary-structure elements (wavy boxes represent helices, arrows represent β strands, and the line represents the coil) and the amino acid residues mutagenized in this study. Residues in bold are those conserved in all WhiB-like proteins. aa, amino acids.

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 *M. smegmatis* 628-53 transformants containing each complementing allele with polyclonal antisera to WhmD. The autoradiograph in Fig. 5A shows that, under conditions of inducer withdrawal, the levels of each of the C-terminal deletion alleles of *whmD* were comparable to the levels of these alleles in WT *whmD* in the positive control, indicating that all these alleles were stable in vivo.

Three of the four conserved cysteine residues and a conserved aspartate are critical to WhmD function. Having broadly mapped the essential boundaries of WhmD, we adopted a site-specific mutagenesis approach to systematically identify residues critical to WhmD activity. The targeted residues included 6 of the 10 residues conserved in all the WhiB-like proteins sequenced to date, as well as a few residues within predicted helices 5 and 6 of the putative DNA binding motif (Fig. 3). To determine the essentiality of the four conserved cysteine residues, we mutagenized each to an alanine residue using the Quik-Change mutagenesis technique and evaluated the ability of each mutant to rescue the filamentation phenotype of the conditionally complemented *whmD* mutant. Converting C_{90}, C_{93}, and C_{99} to alanine was clearly deleterious to WhmD activity, whereas the same did not hold true for C_{67}, the N-terminal cysteine (Fig. 6A, top left; Table 1). The C90A mutant, though filamentous, showed an increased propensity to clump, and the few filaments of which measurements could be made had an average cell length of ~30 μm. The retention of functionality of WhmD C67A was consistent with the observation that the ΔN67 mutant lacking C_{67} was able to functionally complement *M. smegmatis* 628-53. Interestingly, D_{71}, a conserved aspartate residue in close proximity to C_{67}, was found to be essential, since a D71A mutation led to inactivation of WhmD (Fig. 6B, left; Table 1).

Phenotypic effects of helix-destabilizing mutations in putative helices 5 and 6. Complementation analysis of the C-terminal deletion mutants of WhmD suggested that predicted helix 6 was expendable for WhmD function in the filamentation assay. To confirm this hypothesis, we introduced the helix-disrupting mutations R122G and R122P into WhmD and tested the mutants in the filamentation rescue assay. Both mutants remained functional (Table 1), lending credence to the hypothesis of the nonessentiality of this helix in WhmD activity. Moreover, helix 5 was also able to tolerate the helix-disrupting mutations Y102G and Y102P (Table 1).

The L116P mutation mimics the inactive *S. coelicolor whiB70* allele. *whiB70* is an allele of *S. coelicor whiB* which is inactive due to the conversion of a leucine residue to a proline residue at position 74 in WhiB (8). The corresponding leucine residue in WhmD occurs at position 116 and lies just upstream from a predicted α-helical region. In light of the extraordinarily high degree of identity between WhmD and *S. coelicolor* WhiB in their C termini, it is realistic to speculate that the two proteins share conserved structural features. To examine if making a cognate mutation to the *whiB70* allele leads to inactivation of WhmD, L_{116} was mutagenized to a proline residue, and the activity of the mutant was assessed. WhmD L116P was only partially functional (Fig. 6B, right, and Table 1), signifying that the perturbations in structure in both *M. smegmatis* WhmD and *S. coelicolor* WhiB arising as a consequence of this mutation are likely to be similar. We also observed that a G111P mutation which presumably disrupts the β-turn between the two terminal helices also led to WhmD inactivation (Fig. 6B, middle, and Table 1), implying that this structural element is of functional significance.

Western blot analysis indicated that all the tested point mutant alleles were stable in vivo (Fig. 4B), ruling out the possibility that the protein instability of certain alleles was responsible for their inability to complement *M. smegmatis* 628-53.

WhmD structure is sensitive to treatment by a reducing agent. The presence of four conserved cysteine residues sug-

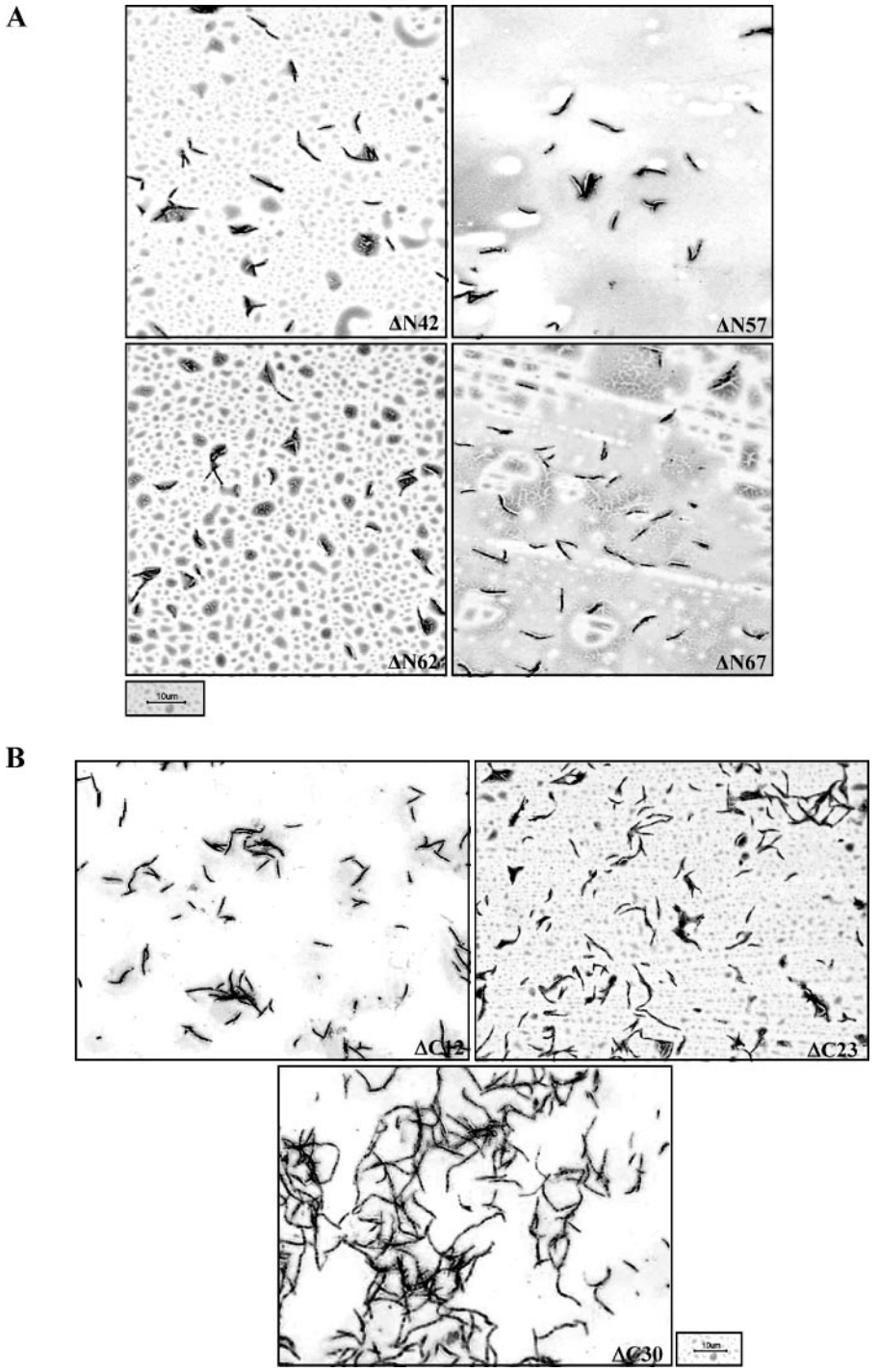


FIG. 4. Complementation phenotypes of N-terminal (A) and C-terminal (B) deletion mutants of WhmD.

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(+) 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 cys-

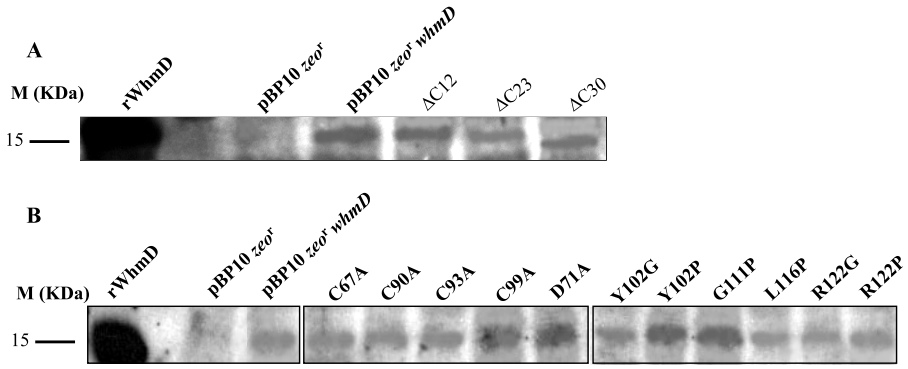


FIG. 5. Estimation of the stability of complementing mutant alleles of WhmD. Western blot analysis of complementing C-terminal deletion mutant alleles (A) and point mutant alleles (B) of WhmD following inducer withdrawal. M, molecular mass marker; rWhmD, purified recombinant WhmD.

teine 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).

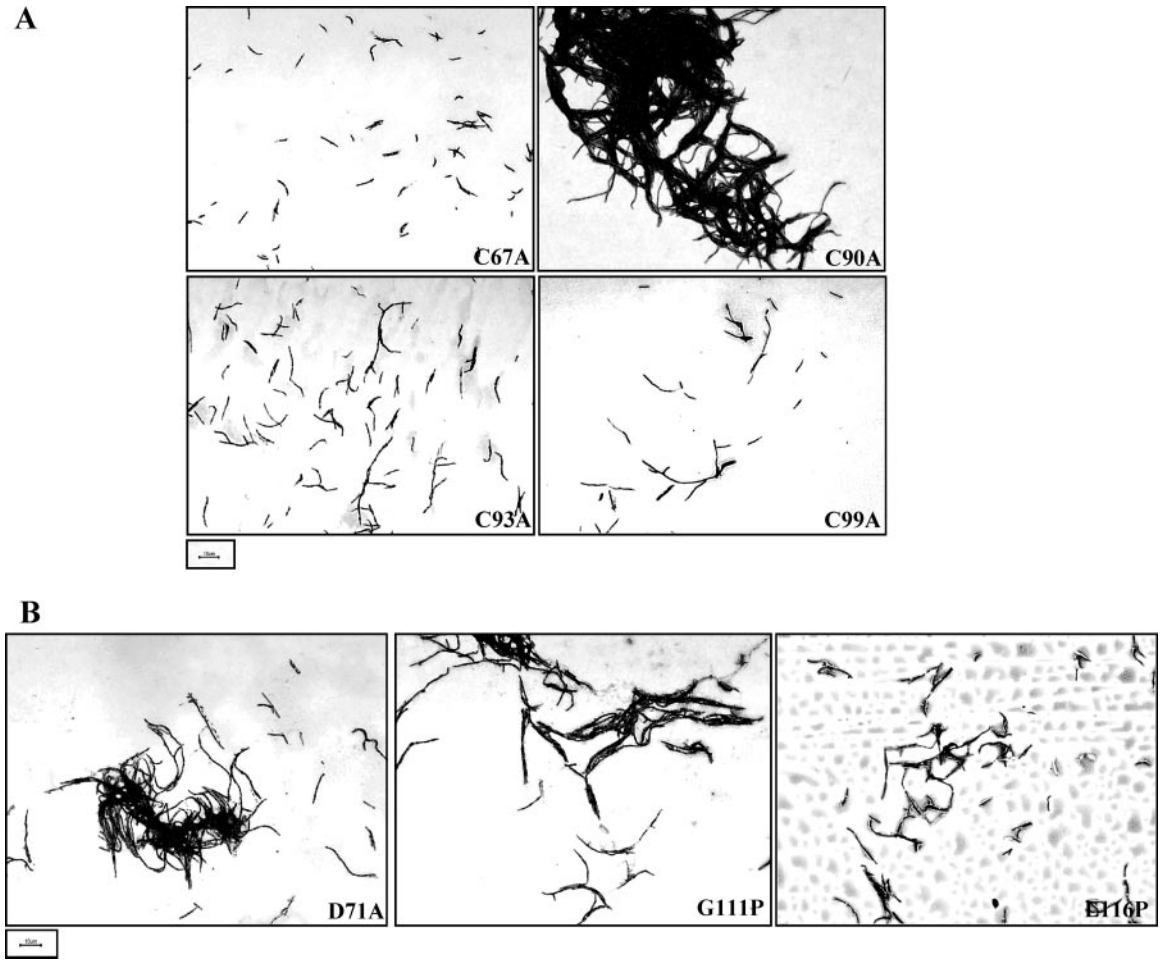


FIG. 6. Phenotypic consequences of mutagenizing conserved residues in WhmD. (A) Complementation phenotypes of the four cysteine-to-alanine mutants of WhmD. (B) The inactivation of a conserved aspartate (left) and a glycine (middle) inactivate WhmD. The L116P mutation mimics the inactive *S. coelicolor whiB70* allele (right).

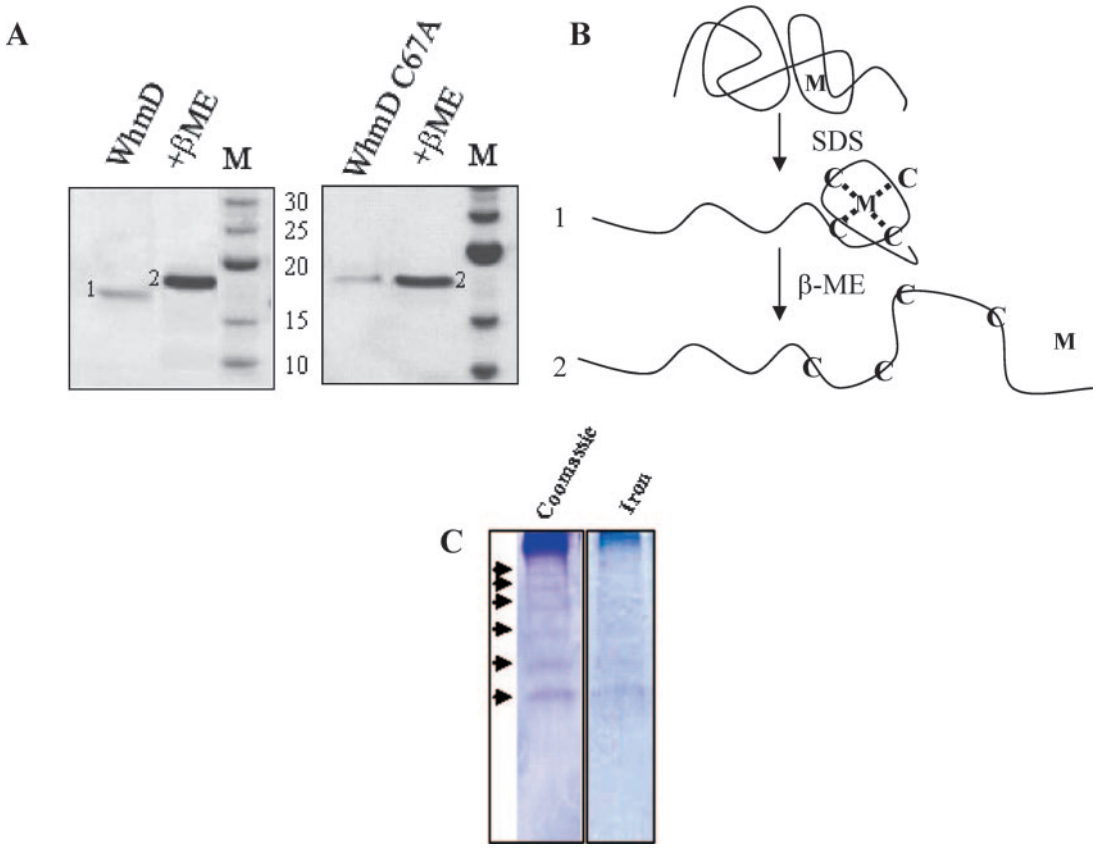


FIG. 7. WhmD structure is sensitive to treatment by a reducing agent. (A) Coomassie staining of purified WhmD (left) or WhmD C67A (right) electrophoresed on a 12% denaturing PAGE gel in the absence (lane 1) and presence (lane 2) of β-mercaptoethanol (β-ME). M, molecular mass markers (kDa). (B) Schematic representation of the effect of the reducing agent on WhmD conformation. Cysteine residues are denoted C, and M is the metal ion. Mobilities of predicted conformations 1 and 2 are indicated in panel A. (C) WhmD contains bound iron. Coomassie blue (left) and Fe-specific (right) staining of WhmD following native gel electrophoresis is shown. Oligomeric forms of WhmD are indicated by the arrowheads.

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 streptomyces (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 C_{67}, 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 C_{67}, it was interesting that WhmD D71A was inactive, implying that this aspartate was functionally essential. It is conceivable that in the absence of C_{67}, D_{71} could play a role in binding the cluster along with C_{90}, C_{93}, and C_{99}, 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 sensor-response regulator system (6) and that D_{71} 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 Asp_{71}, while essential, might be required in order to coordinate a metal ion rather than serve as a phosphate 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 hetero-oligomers 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 structure-function relationships in the WhiB-like protein family.

ACKNOWLEDGMENTS

We gratefully acknowledge the support of NIH grants AI37856, AI36973, AI43846, and AI51668. T.R.R. was the recipient of a postdoctoral fellowship from the Heiser Program.

We thank Keith F. Chater, Helen Kieser, and their colleagues at the John Innes Center, Norwich, United Kingdom, for their invaluable assistance. We thank Rashna Bhandari for helpful discussions and Nisheeth Agarwal for assistance with purification of the cysteine mutants of WhmD.

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