Mycobacterium smegmatis whmD and its homologue *Mycobacterium tuberculosis whiB2* are functionally equivalent

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Mycobacterium smegmatis whmD is an essential gene involved in cell division. This paper shows that *whmD* and its homologue *whiB2* in *Mycobacterium tuberculosis* are functionally equivalent. The genes are syntenous, and share significant homology in both their coding and non-coding DNA sequences. Transcription site mapping showed that the two genes possess near-identical promoter elements, and they displayed comparable promoter strengths in a reporter gene assay. The two proteins show near identity in their C-terminus, and polyclonal antiserum to WhmD specifically cross-reacts with a ~15 kDa band in *M. tuberculosis* lysates. Following overexpression of sense and anti-sense constructs in their cognate mycobacterial hosts, *whiB2* and *whmD* transformants displayed a small-colony phenotype, exhibited filamentation, and showed a reduction in viability. These observations reveal that the two proteins are functionally homologous and that their intracellular concentration is critical for septation in mycobacteria. Colonies of *M. tuberculosis* overexpressing *whiB2* were spherical and glossy, suggesting a change in composition of the cell envelope. Filaments of the conditionally complemented *M. smegmatis whmD* mutant were non-acid-fast, also indicating changes in characteristics of surface lipids. *M. smegmatis* transformants carrying a *whmD-gfp* fusion showed a diffuse pattern of fluorescence, consistent with the putative role of WhmD as a regulator. These observations strongly suggest that *M. tuberculosis whiB2* is an essential gene and its protein product in all likelihood regulates the expression of genes involved in the cell division cascade.

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INTRODUCTION

A prominent virulence trait of *Mycobacterium tuberculosis* is its innately slow growth rate, estimated at 20–24 h in animal models (North & Izzo, 1993), and an adaptive response to host immunity which further slows or even stops its growth during infection. This ability to enter a persistent or latent state within humans allows the organism to await immune deterioration of its host for long periods of time. In addition, clinical observations suggest that the organism develops a state of phenotypic tolerance to antimycobacterial drugs during infection, which may be characterized by a reduced rate of cell division (Colangeli *et al.*, 2005). The basis for the prolonged cell division times is unclear, although the limited number of rRNA operons and the metabolic costs of maintaining the complex cell wall characteristic of the genus *Mycobacterium* are often cited as possible contributing factors (Wheeler & Ratledge, 1994). Of late, significant progress has been made towards an understanding of the temporal and spatial regulation of prokaryotic cell

division. A number of proteins in *Escherichia coli* and *Bacillus subtilis* have been shown to be essential, to be localized to the division septum and to give filamentous phenotypes when conditionally knocked out (Jacobs & Shapiro, 1999).

Although the step-wise assembly pathway involved in formation of the *E. coli* cell division complex has been extensively described (Goehring & Beckwith, 2005) this process is just beginning to be understood in members of the mycobacteria. These studies suggest that FtsZ is an essential cell division protein and that *M. tuberculosis* FtsZ is a target of FtsH protease (Anilkumar *et al.*, 2004, 2001; Dziadek *et al.*, 2002, 2003). Optimal levels of FtsZ are required for cell division, with unregulated expression resulting in lethality (Dziadek *et al.*, 2002). *M. tuberculosis ftsZ* can functionally substitute for its *Mycobacterium smegmatis* orthologue, signifying that the FtsZ-catalysed phases in the cell division processes of slow- and fast-growing members of mycobacteria are analogous (Dziadek *et al.*, 2003). In addition, *M. tuberculosis ftsZ* has been shown to possess multiple promoters, reflecting the requirement to maintain a high basal level of, or to differentially regulate, FtsZ expression

Abbreviations: 5'-RACE, rapid amplification of cDNA ends; 5'UTR, 5' untranslated region.

during different growth conditions of the pathogen *in vivo* (Roy & Ajitkumar, 2005). *M. tuberculosis* FtsZ and the membrane protein FtsW have been shown to interact, *in vitro* (Datta *et al.*, 2002) and *in vivo* (Rajagopalan *et al.*, 2005), suggesting that this interaction could serve to anchor FtsZ to the membrane and link septum formation to peptidoglycan synthesis in *M. tuberculosis*.

Like *ftsZ*, *M. smegmatis whmD* is an essential cell division gene. WhmD belongs to the growing WhiB-like family of proteins shown to be involved in the regulation of significant cellular processes such as cell division (Gomez & Bishai, 2000), pathogenesis (Ramakrishnan *et al.*, 2000; Steyn *et al.*, 2002), antibiotic resistance (Morris *et al.*, 2005) and response to oxidative stress (Kim *et al.*, 2005). Each WhiB-like protein contains four invariant cysteine residues and is relatively short (76–139 residues). Members of this family also possess a conserved C-terminal motif comprising two helices and an intervening turn characterized by the 7-residue signature (FYG)-G-(VI)-W-G-G-(LVIM). Although this motif does not constitute a typical HTH motif (Aravind *et al.*, 2005), it is believed to be involved in DNA binding (Davis & Chater, 1992; Soliveri *et al.*, 2000). In a conditionally complemented system, on inducer withdrawal, a $\Delta whmD$ mutant exhibited irreversible filamentous branched growth with diminished septum formation and aberrant septal placement, while WhmD overexpression resulted in growth retardation and hyperseptation (Gomez & Bishai, 2000). Together these phenotypes indicated a role for this protein in septum formation and cell division. The septal defects were found not to be due to insufficient levels of FtsZ, since FtsZ accumulation did not vary following WhmD withdrawal. WhmD is therefore proposed to play a role in the early stages of mycobacterial cell division, perhaps in FtsZ localization or polymerization or as a regulator of cell division genes other than *ftsZ* (Gomez & Bishai, 2000). No functional information is available on *whiB2*, the homologue of *whmD* in *M. tuberculosis*, apart from its induction in a nutrient-starvation model of *M. tuberculosis* (Betts *et al.*, 2002) and in the lungs of infected mice (Dubnau *et al.*, 2005). The two genes share synteny at the genomic level and almost 70% identity in their protein sequence. Furthermore, whereas the C-termini of WhmD and WhiB2 are nearly identical to that of *Streptomyces coelicolor* WhiB, the mycobacterial proteins contain a unique 38–48 aa N-terminal extension not found in WhiB. Although *whiB2* is annotated as an 89-codon ORF (Cole *et al.*, 1998) based on homology to *whiB*, the protein is believed to be translated from a start codon 102 nt upstream of that predicted (Gomez & Bishai, 2000). To examine if WhmD and WhiB2 are true homologues, we have carried out a comparative analysis of the two genes at the level of their transcripts, protein products and the phenotypic effects of their perturbation. Our results indicate that the two proteins are functionally equivalent, and strongly suggest that *M. tuberculosis whiB2* is an essential gene involved in mycobacterial cell division.

METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* DH5 α (F⁺*endA1 hsdR17* [*r_k⁻ m_k⁺*] *glnV44 thi-1 recA1 gyrA* [Nal^r] *relA1* Δ [*lacZYA-argF*]U169 *deoR* [ϕ 80*dlac* Δ (*lacZ*)M15]), from Stratagene, 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. *M. smegmatis* mc²6 1-2c was kindly provided by Dr Bill Jacobs, Albert Einstein College of Medicine, NY, USA, and *M. tuberculosis* CDC1551 and its genomic DNA were obtained from Colorado State University, CO, USA. Luria–Bertani (LB) broth and LB agar were used to culture *E. coli*. For culturing *M. smegmatis*, 7H9 broth and 7H10 agar from Difco were supplemented with albumin dextrose complex (ADC; 5 g BSA, 2 g glucose and 0.85 g NaCl l⁻¹), 0.5% (v/v) glycerol and 0.05% Tween 80. For *M. tuberculosis*, ADC was substituted with Middlebrook OADC (oleic acid/albumin/dextrose/catalase) enrichment (Becton Dickinson). Both *E. coli* and mycobacteria were grown at 37 °C with shaking at 200 r.p.m. Antibiotics were added when necessary: ampicillin (200 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹ for *E. coli* and 15 μ g ml⁻¹ for mycobacteria), hygromycin (200 μ g ml⁻¹ for *E. coli* and 50 μ g ml⁻¹ for mycobacteria), zeocin (25 μ g ml⁻¹ for *E. coli* and 50 μ g ml⁻¹ for mycobacteria) and apramycin (30 μ g ml⁻¹).

DNA techniques. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (NEB), and *Taq* polymerase was purchased from Invitrogen. 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, and *E. coli* transformation were performed as described by Sambrook *et al.* (1989). Mycobacterial strains were transformed by electroporation. PCR amplifications were carried out according to the manufacturer's specifications (Bio-Rad). 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 labelling reactions were purified by using the Qiagen gel extraction kit according to the manufacturer's specifications.

Western blotting. For immunoblotting experiments, *M. tuberculosis* and *M. smegmatis* cells were harvested at their exponential phase of growth, lysed by bead beating on a mini bead beater (Biospec Products) and protein was quantified by the Bradford assay (Bradford, 1976). Samples containing 30 μ g of total cell lysate proteins were electrophoresed on a 12% SDS-PAGE system and the proteins transferred to nitrocellulose. WhmD (Gomez & Bishai, 2000) and Gfp antisera (Invitrogen) were used at 1:100 and 1:5000 dilutions, respectively. Horseradish-peroxidase-conjugated goat anti-rabbit IgG at a 1:5000 dilution and chemiluminescent substrate (Amersham) were used to develop the blots.

Mapping the 5' ends of *whmD* and *whiB2* mRNA. The 5'-RACE (rapid amplification of cDNA ends) system (Frohman, 1993) was used to determine the transcription initiation site with the Invitrogen kit (version 2.0). Total RNA was isolated from *M. smegmatis* and *M. tuberculosis* cells harvested at their exponential phase of growth by using the Trizol method according to the supplier's instructions (Invitrogen). The abridged anchor primer (AAP) and abridged universal amplification primers (AUAP) were used in combination with the gene-specific primers. The gene-specific primer *whmD* RACE gsp1 (5'-ACGTCCAGCTGCCCCGTCG-3') was used for RT-PCR and nested PCR1 while *whmD* RACE gsp2 (5'-GGCTCACCAGCCGAGTAGC-3') was used for the second nested PCR with AUAP. Likewise, *whiB2* RACE gsp1 (5'-CGGCC-CTCGGGAACCAAC-3') was used for the RT-PCR and first nested PCR step, while *whiB2* RACE gsp2 (5'-ATGCGGTAGCCGATCCGGTA-3') was used for nested PCR 2. The PCR products were subsequently subjected to nucleotide sequencing by using the gene-specific primers *whmD* RACE gsp2 and *whiB2* RACE gsp2.

Construction of *lacZ* transcriptional fusions and measurement of β -galactosidase activity. To generate P_{whmD} -*lacZ*, a 187 bp DNA fragment containing the *whmD* promoter sequence from positions -187 to -1 with respect to the start codon (ATG), was PCR-amplified from *M. smegmatis* chromosomal DNA using the primers P_{whmD5B} -F (5'-CTAGTCTAGAGAATTCGCGCCCTG-GAGC-3') and P_{whmD5B} -R (5'-ACATGCATGCATCCCCGCCT-CCTCACT-3'). The transcriptional fusion construct was generated by cloning the amplified fragment in pSD5B at the *XbaI* and *SphI* sites. P_{whiB2} -*lacZ* was constructed by PCR amplification of a 200 bp fragment from *M. tuberculosis* genomic DNA using the primers pSD5B $whiB2$ -F (5'-CTAGTCTAGATTACGAGATGATATGGAA-3') and pSD5B *MtwhiB2*-R (5'-ACATGCATGCGCCTCCGCCTC-CTCACTC-3') and cloning the fragment into pSD5B at the *XbaI*-*SphI* sites. The positive control fusion, P_{hsp60} -*lacZ*, was made by amplifying a 385 bp fragment containing the *M. bovis hsp60* promoter from the vector pMV261 using the primers pSD5B $hsp60$ -F (5'-CTAGTCTAGAAATCTAGACGGTGACCA-3') and pSD5B $hsp60$ -R (5'-ACATGCATGCTGCGAAGTGATTCTCCG-3') followed by cloning the fragment at the *XbaI*-*SphI* sites of pSD5B. All the above constructs and the control plasmid pSD5B were introduced into *M. smegmatis* by electroporation, and promoter activity was determined by β -galactosidase assays (Miller, 1972), using cell lysates of the cultures harvested at their exponential phase of growth. Protein concentrations were determined by the Bradford assay, with BSA as the standard. At least three biological replicates were performed for each sample before calculating the specific activity.

Expression of sense and anti-sense constructs of *whmD* and *whiB2* and examination of their phenotypes. Sense and anti-sense constructs of *whmD* and *whiB2* were generated by PCR-amplifying the entire ORFs of the two genes from genomic DNA of their cognate hosts in the sense and anti-sense orientations, and cloning the products into the *NdeI*-*SpeI* sites of pCK0218 (Manabe *et al.*, 1999). This results in the replacement of the *sigF* gene in the plasmid with the above products, allowing the regulation of their expression in response to acetamide. The primers used to amplify the two genes were as follows. For pAce *whmD*: pAce $whmD$ -F (5'-GGGAATTCATATGTCTTATGAGACGGCGAT-3'), pAce $whmD$ -R (5'-GGACTAGTCTAGATGATGCGCGCTT-3'). For pAce anti-*whmD*: pAce anti-*whmD*-F (5'-GGGAATTCATATGCTAGATGATGCGCGCTT-3'), pAce anti-*whmD*-R (5'-GGACTAGTATGTCT-TATGAGACGGC-3'). For pAce *whiB2*: pAce $whiB2$ -F (5'-GGG-AATTCATATGTCCTATGAACACCTTCGG-3'), pAce $whiB2$ -R (5'-GGACTAGTTCAGATGATCCCGGCTT-3'). For pAce anti-*whiB2*: pAce anti-*whiB2*-F (5'-GGGAATTCATATGTCAGATGAT-CCCGGCTT-3'), pAce anti-*whiB2*-R (5'-GGACTAGTATGTCCT-ATGAACACCTT-3'). The control plasmid pAce was constructed by digesting pCK0218 with *NdeI*/*SpeI*, purifying the vector fragment, endfilling the fragment with the Klenow fragment of DNA polymerase and ligating the vector to itself. To alter the levels of *WhmD*/*WhiB2*, the recombinant plasmids described above were transformed into their cognate mycobacterial hosts along with the control plasmid pAce. Transformants were grown to their exponential phase of growth ($OD_{600} \sim 1.0$), and then grown overnight in the presence or absence of 0.2% acetamide. For colony phenotypes and viability measurements, appropriate culture dilutions were plated on 7H10 agar medium either containing or lacking the inducer. For induced cultures, it was necessary to maintain a concentration of 0.2% acetamide on agar plates. To document colony phenotypes, plates were photographed using a Fuji-Finepix A340 digital camera and the images edited using the Adobe Elements 2.0 software package. Percentage viability was calculated by dividing the number of c.f.u. obtained under inducing conditions by that observed in the absence of the inducer. The data represent at least two independent replicates. To observe cellular morphology, cultures were washed and resuspended in PBS, heat fixed on slides and stained with carbolfuchsin

for 5 min. After washing off the excess dye with distilled water, the slides were dried and examined at $600\times$ magnification on a Nikon Eclipse E800 microscope under oil. Images were captured using the Nikon digital still camera DXM1200 and edited using the software package ACT-1 ver2. Cell length measurements were made using a sample size of 30.

Real-time RT-PCR analysis. To quantify transcript levels of *whmD* and *whiB2* in their cognate hosts under conditions where the levels of the genes were being perturbed, RNA was isolated from the acetamide-induced cultures (as described above), treated with RNase-free DNase (Ambion) and subjected to reverse transcription. This was followed by real-time quantitative PCR using SYBR Green Supermix (Bio-Rad). *whmD* was amplified using the primers *whmD* RT-F (5'-GTGAGCCATGCACCGCAC-3') and *whmD* RT-R (5'-CGCTTGGCCTCTCGGGTG-3'). The primers *whiB2* RT-F (5'-GCGCCATTCGAGGAACCT-3') and *whiB2* RT-R (5'-CAGATGCC-GAACCGCTCG-3') were used to amplify *whiB2*. Both sets of primers amplify 200 nt of the respective gene. The relative fold change of mRNA of the two genes under each of the experimental conditions was measured by normalizing its transcript level to that of sigma factor A (*sigA*) [amplified by using primers *sigA*-F (5'-CGATGACGACGAGGAGATCGC-3') and *sigA*-R (5'-CAGCGCT-ACCTTGCCGATCTG3')]. Data from three independently derived RNA samples were used to determine mean fold increase in transcript levels.

Acid-fast staining of *M. smegmatis* 628-53. *Ms* 628-53, the conditionally complemented *whmD* mutant, was cultured in 7H9 broth supplemented with 0.2% acetamide, grown to an OD_{600} of 1.0 and washed twice with 7H9 broth. The cells were then resuspended in 7H9 broth, split into two and grown overnight (~ 16 h) in the presence and absence of 0.2% acetamide. The cultures were washed and resuspended in PBS, and heat-fixed on slides. Staining was carried out with the Difco acid-fast staining kit. Briefly, the smears were first stained with carbolfuchsin for 5 min followed by rinsing off the primary stain with water. The slide was then drained and de-colored with acid-alcohol until no more stain appeared. Counterstaining was carried out with TB brilliant green for 1 min. After washing off the excess dye with distilled water, the slides were dried and examined at $600\times$ magnification on a Nikon Eclipse E800 microscope under oil. Images were captured using the on-board digital still camera DXM1200 and manipulated using the software package ACT-1 ver2.

Construction of the *whmD*-*gfp* fusion and fluorescence microscopy. To generate pWG, the vector containing the *whmD*-*gfp* translational fusion, *whmD* was first amplified with its promoter region from *M. smegmatis* genomic DNA using the primers P_{whmD27} -F (5'-AGCGATATCGAATTCGCGCCCTGGAGC-3') and *whmD* tag-R (5'-CGGGGTACCGATGATGCCGCGCTTGAG-3'). The *gfp* gene was then PCR-amplified from the vector pFPV27 (Ramakrishnan *et al.*, 2000) using the primers *gfp* Δ atg-F (5'-CGG-GGTACCTCTAAAGGTGAAGAAT-3') and *gfp*27-R (5'-ACATGCA-TGCTTATTTGTACAATTCATC-3'). The *whmD* fragment digested with *EcoRV*/*KpnI* was then ligated to *KpnI*/*SphI*-digested *gfp* amplified as above and *EcoRV*/*SphI*-digested pFPV27 in a three-way ligation. The resultant recombinant contained *whmD* expressed from its own promoter, translationally fused to *gfp* in the pFPV27 backbone. pFL-*whmD* was constructed by PCR-amplifying *whmD* with its promoter region from *M. smegmatis* chromosomal DNA with the primers P_{whmD27} -F and *whmD*27-R (5'-ACATGCATGCCTAGAT-GATzGCCGCGCTT-3') and cloning the fragment into the *EcoRV*-*SphI* sites of pFPV27. pWG, pFL $whmD$ and the control plasmids pFPV27 and pBEN were transformed into *M. smegmatis* mc²6 1-2c and the transformants cultured to their exponential phase of growth. The cells were then harvested, washed and resuspended in PBS. A drop of this suspension was put on a glass slide under a

Fig. 1. Size estimation of *M. tuberculosis* WhiB2. (a) Sequence alignment of *M. smegmatis* WhmD and WhiB2, its *M. tuberculosis* orthologue. (b) Western blot analysis of *M. smegmatis* (Ms) and *M. tuberculosis* (Mt) lysates using polyclonal antiserum to WhmD.

cover slip, and Gfp fluorescence visualized at $600\times$ magnification on a Nikon Eclipse E800 microscope under oil. Images were captured using the SPOT epi-fluorescence camera and manipulated using the SPOT software package.

***M. smegmatis* 628-53 complementation assays.** To generate pBP10WG, the *whmD-gfp* fusion construct was amplified from pWG using the primers pBP10*whmD*-F (5'-AAACTGCAGGA-ATTCGCGCCCTGGAGC-3') and pBZ*gfp*-R (5'-GGACTAGTTTATTTGTA caattcatc-3') and cloned at the *Pst*I-*Spe*I sites of pBP10 *zeo*. The plasmid pBP10 *zeo whmD* was generated by cloning a wild-type copy of *M. smegmatis whmD* into pBP10 *zeo* (Raghunand & Bishai, 2006) to serve as a positive control for complementation. For complementation analysis, pBP10 *zeo whmD-gfp* (WG), pBP10 *zeo whmD* (W) and the parent plasmid pBP10 *zeo* (V) were transformed into *Ms* 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 OD₆₀₀ 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 PBS, heat-fixed on slides and stained with carbol-fuchsin for 5 min. After washing off the excess dye with distilled water, the slides were dried and examined at $600\times$ or $1000\times$ magnification on a Nikon Eclipse E800 microscope under oil. Images were captured using the on-board digital still camera DXM1200 and edited using the software package ACT-1 ver2. The cell lengths reported represent the mean $\pm$ SEM of 30 cells.

Sequence analysis. All sequence alignments were performed on the BCM Search Launcher, Multiple Sequence Alignment package (Baylor College of Medicine) using the ClustalW 1.8 algorithm. The output files were imported into Boxshade 3.21 (<http://www.ch.embnet.org>) to generate the formatted alignments shown in Figs. 1 and 2. The boundary coordinates of the 5'UTR sequences shown in Fig. 1(c) with reference to their locations upstream to the start codon are as follows: *Ms whmD*, -187 to -1; and *Mt whiB2*, -185 to -1.

RESULTS AND DISCUSSION

Clarifying the discrepancy in genome annotation vs sequence alignment: size estimation of WhiB2

whiB2 has been assigned conflicting annotations in the databases containing sequence information on the two strains of *M. tuberculosis* completely sequenced so far. The Tuberculist (<http://genolist.pasteur.fr/TubercuList/>) database containing sequence information from *M. tuberculosis* H37Rv catalogues *whiB2* (Rv3260) as a 270 bp gene encoding an 89 aa protein. In contrast, this gene is annotated as a 370 bp gene encoding a 123 aa protein (MT3358) in the *M. tuberculosis* CDC1551 genome sequence (<http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org=gmt>), and sequence alignment of WhmD and WhiB2 (Fig. 1a) seems to support the accuracy of this annotation. The 89 aa putative polypeptide is predicted to have a molecular mass of 9.7 kDa, while that for the 123 aa protein is 13.5 kDa. To address this discrepancy, we carried out immunoblot analysis of *M. smegmatis* and *M. tuberculosis* lysates using polyclonal antiserum to WhmD. This antiserum is specific for WhmD despite the presence of other WhiB-like proteins in mycobacteria. The homology within the WhiB paralogues is limited, apart from the conservation of the WhiB signature, so this antibody was not expected to cross-react strongly with any of the other members of the family. As shown in Fig. 1(b), the antibodies specifically react with a protein of approximately 14 kDa in *M. smegmatis* lysates (lane 1) as documented before (Gomez & Bishai, 2000), and cross-react with a protein of similar size in *M. tuberculosis* lysates (lane 2). No cross-reactivity was observed

Fig. 2. Transcription site mapping of *M. smegmatis whmD* and *M. tuberculosis whiB2*. (a) Amplification products from 5'-RACE analysis of *whmD* and *whiB2*. M, molecular mass markers (1 kb Plus DNA ladder, Invitrogen). (b) Sequencing electropherogram traces of 5'-RACE amplicons of *whiB2* and *whmD*. (c) Alignment of DNA sequences of promoters of *whmD* and *whiB2*, showing the -10 and -35 promoter elements, the transcription start sites (arrow), and the Shine-Dalgarno (SD) sequences.

in the 9–13 kDa regions where the *M. smegmatis* orthologues of WhiB1, WhiB3, WhiB6 and WhiB7 would be expected to migrate. These results strongly suggest that translation of WhiB2 originates from a start codon far upstream of that originally predicted, resulting in a protein product 34 aa longer. The use of this site is also corroborated by the presence of a consensus Shine–Dalgarno sequence (AGGAGG), 7 nt upstream of the WhiB2 start codon (Fig. 2c). We therefore propose that WhiB2 is a 123 aa protein, consistent with the prediction based on sequence alignment of the homologues.

Conservation of promoter elements: transcription of *whmD* and *whiB2* initiates from the same nucleotide

Having established that the protein products of *whmD* and *whiB2* are similar in length, we examined whether the transcriptional elements driving the expression of these

genes are conserved as well. In order to delineate the promoter region, we determined the transcription start site of both transcripts by the 5'-RACE technique, as described in Methods. Total RNA was isolated from *M. smegmatis* and *M. tuberculosis* at their mid-exponential phase of growth and final amplification of dC-tailed cDNA was performed using the primer AUAP and gene-specific primers to *whmD* (*whmD* RACE gsp2) and *whiB2* (*whiB2* RACE gsp2) which anneal 100 nt downstream of the start codon. As shown in Fig. 2(a), the size of the amplicons indicated that the start site lies 200–300 bp upstream of the gsp2 annealing site. Sequencing of the amplified products (Fig. 2b) indicated that *whmD* and *whiB2* transcription initiates from an A residue 134 and 133 nt upstream of their respective start codons. Sequence analysis of the region upstream of the transcription start site revealed the presence of consensus *E. coli* promoter-like sequences at the -35 and -10 positions (Fig. 2c). The two genes share identical -35 elements, whereas the -10 elements are mismatched by a single base.

Fig. 3. (a) Schematic representation of the control plasmid (pAce) and the constructs used to overexpress *whmD* and *whiB2* in their sense and anti-sense orientation. (b, c) Graphical representation of the fold change in *whmD* transcript levels following expression of sense (S) and anti-sense (A) constructs of *whmD* in *M. smegmatis* (Ms, b) and of *whiB2* in *M. tuberculosis* (Mt, c) under inducing conditions (+). In both graphs the transcript levels of the respective genes in vector-transformed cells (V) are assigned a value of 1. The error bars represent SEM.

The promoters driving the expression of *whmD* and *whiB2* have similar strengths

The extensive conservation in their 5'UTRs and the near-identical promoter elements of *whmD* and *whiB2* led us to postulate that the transcription of the two genes was driven by promoters of comparable strength. To verify this hypothesis, we determined the activity of the two promoters using a β -galactosidase-based reporter system. Fragments consisting of 187 and 200 bp of the sequence upstream of the start codons of *whmD* (P_{whmD}) and *whiB2* (P_{whiB2}), respectively, were cloned upstream to a promoterless *lacZ* gene in pSD5B, a mycobacterial promoter-probe vector (Jain *et al.*, 1997). A recombinant plasmid containing a 385 bp fragment from the *Mycobacterium bovis* *hsp60* promoter (Stover *et al.*, 1991) and the empty vector were used as positive and negative controls, respectively. All plasmids were transformed into *M. smegmatis* and promoter activity was estimated by β -galactosidase assays with cell lysates from transformants harvested in their exponential phase of growth. P_{whmD} (185.53 ± 4.79 nmol min⁻¹ mg⁻¹) and P_{whiB2} (110.73 ± 8.16 nmol min⁻¹ mg⁻¹) showed

comparable activities, substantially higher than the control plasmid (12.99 ± 0.65 nmol min⁻¹ mg⁻¹). However, the two promoters were considerably weaker than the *hsp60* promoter, which showed a β -galactosidase activity of 1107.16 ± 39.1 nmol min⁻¹ mg⁻¹. Although the activities of the two promoters were significantly higher than the baseline, they could be categorized as weak promoters, based on the comparison to the activity observed with the *M. bovis* *hsp60* promoter. We surmise, based on the observed promoter strengths, that the two genes are expressed at low levels in their respective hosts. Since most DNA-binding proteins are expressed at low levels *in vivo*, these observations of low-level reporter gene expression are consistent with the premise that WhmD and WhiB2 are regulatory proteins.

Physiological consequences of perturbing cellular levels of WhmD and WhiB2

whmD has been shown to be an essential gene in *M. smegmatis*, and lack of the gene product leads to severe defects in septation (Gomez & Bishai, 2000). To determine if

Fig. 4. Phenotypic effects of perturbation of WhmD levels in *M. smegmatis*. (a) Colony phenotypes of *M. smegmatis* transformed with control pAce-V (V), pAce-*whmD* (S) or pAce-anti-*whmD* (A) on 7H10 plates containing (+) or lacking (–) 0.2% acetamide. (b) Cellular morphology of pAce-V (V), pAce-*whmD* (S) or pAce-anti-*whmD* (A) transformants of *M. smegmatis* mc²6 under inducing conditions. Cells were stained with carbolfuchsin and visualized by light microscopy. Bar, 10 μ m. (c) Viability of *M. smegmatis* transformants containing pAce (vector, V), pAce-*whmD* (sense *whmD*, S) or pAce-anti-*whmD* (antisense *whmD*, A).

Table 1. Cell length measurements of transformants of *M. smegmatis* (a) and *M. tuberculosis* (b) expressing sense and anti-sense constructs of *whmD* and *whiB2*, respectively, under inducing conditions

Control transformants contained the vector pAce alone. The table shows the number of cells of a particular length. $n=30$.

(a) Construct 1–2 μm 2–4 μm 5–7 μm					Mean $\pm$ SEM
Vector	19	9	2		2.45 $\pm$ 0.2 μm
Sense	–	19	11		4.42 $\pm$ 0.28 μm
Anti-sense	4	16	10		4.12 $\pm$ 0.29 μm
(b) Construct 2–3 μm 3–5 μm 5–8 μm 9–15 μm					Mean $\pm$ SEM
Vector	17	13	–	–	3.46 $\pm$ 0.19 μm
Sense	–	13	12	5	6.64 $\pm$ 0.54 μm
Anti-sense	–	12	15	3	6.14 $\pm$ 0.39 μm

whiB2, the orthologue of *M. smegmatis whmD*, plays a functionally equivalent role in *M. tuberculosis*, we examined the effects of artificially perturbing *whmD* and *whiB2* levels in their cognate hosts. If the consequences of this perturbation were similar in the two organisms, this would be a pointer to the two genes being operational counterparts. To alter normal cellular levels of the two proteins, *whmD* and *whiB2* were cloned in the sense and anti-sense orientations, under the control of the acetamide-regulatable promoter *P_{ace}* (Parish *et al.*, 1997) in pAce, a derivative of the plasmid pCK0218 (Manabe *et al.*, 1999) (Fig. 3a). The plasmids were transformed into their cognate mycobacterial hosts and RNA was extracted from these transformants grown under inducing conditions. Quantitative RT-PCR analysis was used to confirm that all the transformants showed the anticipated changes in *whmD/whiB2* transcript levels (Fig. 3b, c). The phenotypic effects of altering WhmD/WhiB2 levels were assessed by examining the transformants for colony size, cellular morphology and viability under inducing conditions.

Fig. 5. Phenotypic effects of perturbation of WhiB2 levels in *M. tuberculosis*. (a) Colony phenotypes of *M. tuberculosis* transformed with control pAce-V (V), pAce-*whiB2* (S) or pAce-anti-*whiB2* (A) on 7H10 plates containing (+) or lacking (–) 0.2% acetamide. (b) Cellular morphology of pAce-V (V), pAce-*whiB2* (S) or pAce-anti-*whiB2* (A) transformants of *M. tuberculosis* under inducing conditions. Cells were stained with carbolfuchsin and visualized by light microscopy. Bar, 10 μ m. (c) Viability of *M. tuberculosis* transformants containing, pAce (vector, V), pAce-*whiB2* (sense *whiB2*, S) or pAce-anti-*whiB2* (antisense *whiB2*, A).

M. smegmatis transformants overexpressing either the sense or the anti-sense constructs of *whmD* showed a small colony phenotype (Fig. 4a, panels S+, A+) while control transformants containing the empty plasmid alone in the presence of acetamide induction showed no such defect (Fig. 4a, panel V+). In addition, the *M. smegmatis whmD* sense and anti-sense transformants also exhibited elongation/filamentation, as assessed by carbolfuchsin staining followed by light microscopy (Fig. 4b, panels S+, A+), while control transformants showed normal cellular morphology (Fig. 4b, panel V+). Cell length measurements showed that transformants containing either the sense or the anti-sense construct were significantly elongated compared to controls (Table 1a). Estimation of viability by comparing c.f.u. of transformants grown under uninduced vs induced conditions revealed that overexpression of either construct led to a decrease in viability of *M. smegmatis* as compared to controls (Fig. 4c). The loss in viability on overexpression of *whmD* can be associated with the presence of multiply septate bacteria seen in a *whmD*-overexpressing strain (Gomez &

Bishai, 2000), since such cells are unlikely to contribute to c.f.u. counts. All the observed phenotypes are expected, since *whmD* is an essential cell division gene, and reduction in WhmD levels is lethal to normal cellular physiology. In keeping with this premise, expression of anti-sense constructs of *dnaA*, an essential DNA replication gene had similar consequences in *M. smegmatis* (Greendyke *et al.*, 2002).

The phenotypic effects of overexpression of sense and anti-sense constructs of *whiB2* in *M. tuberculosis* were markedly similar to those observed in *M. smegmatis* transformants with altered WhmD levels. *M. tuberculosis* transformants with elevated or reduced levels of *whiB2* were smaller than controls (Fig. 5a, panels S+, A+ vs V+), displayed significant filamentation (Fig. 5b, panels S+, A+ vs V+, Table 1b) and showed reduced viability as compared to control transformants (Fig. 5c). In *M. tuberculosis*, anti-sense overexpression of *whiB2* resulted in a ~90% loss of viability, strongly suggesting that *whiB2* is an essential gene in *M. tuberculosis*.

Fig. 6. Cell envelope changes associated with altered WhiB2/WhmD levels in their cognate hosts. (a) Colony phenotypes of *M. tuberculosis* transformed pAce-*whiB2* (S) on 7H10 plates containing (+) or lacking (–) 0.2% acetamide. (b) Acid-fast staining properties of *Ms* 628-53, a conditionally complemented *M. smegmatis whmD* mutant, under permissive (0.2% acetamide, Ac) and non-permissive (–Ac) conditions.

The differences in cell lengths and viability in *M. tuberculosis* vs *M. smegmatis* could be a consequence of the differential expression of the sense and anti-sense plasmid constructs. Taken together, these observations provide compelling evidence that *M. smegmatis whmD* and *M. tuberculosis whiB2* are functional homologues. Moreover, these findings also indicate that the levels of the WhmD and WhiB2 proteins are crucial for normal mycobacterial septation, and any imbalance in their levels can lead to atypical cellular morphology and loss of viability. Not surprisingly, the same observation was made for the mycobacterial FtsZ protein (Dziadek *et al.*, 2002).

Cell envelope changes associated with alteration of WhiB2 or WhmD concentrations in their cognate hosts

On closer observation of the morphology of *M. tuberculosis* transformants overexpressing *whiB2*, we found that these colonies were glossy and spherical, whereas the corresponding controls were rough and flat (Fig. 6a, S+ vs S–). This phenotype closely resembles the appearance of the *fbpA* mutant of *M. smegmatis*, where the change in colony morphology was attributed to a decrease in production of α,α' -trehalose dimycolate (Nguyen *et al.*, 2005). This raises the possibility that WhiB2 may be associated with regulating the expression of components of the *M. tuberculosis* cell envelope. However, no such change was observed in transformants expressing the anti-sense construct of

whiB2. Curiously, the conditionally complemented *M. smegmatis whmD* mutant *Ms* 628-53 (Gomez & Bishai, 2000) was observed to be non-acid-fast under conditions of acetamide withdrawal (Fig. 6b). It is known that the carboxyl and hydroxyl groups of cellular lipids like mycolic acid are essential to the acid-fast reaction of mycobacteria (Harada, 1976), which could imply that in the absence of WhmD there are changes in modification of the surface lipids of *M. smegmatis*. These two observations seem to point to the involvement of these proteins in cell-envelope-related transactions in their respective hosts.

Gfp fluorescence in a *whmD*–*gfp* fusion localizes to the cytoplasm of *M. smegmatis* cells

The WhiB-like proteins are believed to be DNA-binding regulatory proteins. However, the septation defect and filamentation observed in *M. smegmatis* cells lacking WhmD (Gomez & Bishai, 2000) led us to hypothesize that WhmD perhaps plays a structural role in cell division by localizing to developing septa. To determine its subcellular localization, *whmD* was fused to the gene encoding green fluorescent protein (Gfp), in the vector pFPV27 (Ramakrishnan *et al.*, 2000), with the fusion protein being expressed from the *whmD* promoter. A construct carrying the full-length *whmD* gene, and the empty vector, were used as negative controls. The plasmid pBEN (Saviola *et al.*, 2003) expressing *gfp* from

Fig. 7. GFP fluorescence in a *whmD*–*gfp* fusion localizes to the cytoplasm of *M. smegmatis* cells. (a) Schematic representation of the constructs used in the fluorescence study. pFPV27 and pFL-*whmD* are the negative control plasmids, pBEN is the positive control, and pWG is the test plasmid containing the *whmD*–*gfp* fusion. (b) Western blots of *M. smegmatis* lysates containing the *whmD*–*gfp* fusion plasmid (mc²6:pWG) or the parent plasmid (mc²6:pFPV27), with polyclonal antiserum against Gfp. The arrow marks the 41 kDa WhmD–Gfp fusion protein. (c) Morphology of vector-transformed (V), *whmD*-transformed (W) or *whmD*–*gfp* (WG)-transformed *Ms* 628-53 on inducer withdrawal. Bar, 10 μ m. (d) GFP fluorescence of *M. smegmatis* transformants containing the plasmids depicted in (a).

the *hsp60* promoter (Stover *et al.*, 1991) served as the positive control for Gfp fluorescence (Fig. 7a). The expression of the *whmD-gfp* fusion was confirmed by RT-PCR analysis (data not shown) and Western blotting using polyclonal antisera to GFP (Fig. 7b). To assess the functionality of the fusion protein, the *whmD-gfp* construct was cloned into pBP10 *zeo* (Raghunand & Bishai, 2006), a derivative of pBP10 (Bachrach *et al.*, 2000), transformed into *Ms* 628-53, a conditionally complemented *whmD* mutant (Gomez & Bishai, 2000), and its complementation phenotype evaluated in a filamentation rescue assay (Raghunand & Bishai, 2006). Under non-permissive conditions, *Ms* 628-53 containing the control plasmid showed extensive filamentation, with a mean cell length of 11.99 ± 0.56 μm . Transformants containing both the *whmD-gfp* fusion (mean cell length 4.0 ± 0.27 μm) and a WT *whmD* construct (mean cell length 3.69 ± 0.22 μm) were able to rescue this phenotype (Fig. 7c), establishing that the fusion construct was indeed functional. Upon Gfp fluorescence microscopy, no fluorescence was seen in cells containing the negative control plasmids pFPV27 and pFL*whmD*, as expected. Transformants of *M. smegmatis* expressing the WhmD-Gfp fusion from the plasmid pWG displayed a diffuse pattern of fluorescence, mirroring that observed with pBEN (Fig. 7d). No subcellular localization or distinct foci of fluorescence were detected in cells in any of the several fields examined, which suggests a regulatory role for WhmD in cell division as opposed to a structural role where WhmD would form a part of the septation complex. WhmD and WhiB2 are therefore assumed to influence the process of cell division by regulating the expression of genes directly or indirectly involved in septation. The identity of these genes is currently unknown. In the recent past, defects in several different gene products have been associated with mycobacterial growth and morphology. These include the eukaryotic-like serine/threonine kinases PknA (Chaba *et al.*, 2002; Kang *et al.*, 2005), PknB (Kang *et al.*, 2005) and PknF (Deol *et al.*, 2005) as well as the gene encoding phosphomannose isomerase (Patterson *et al.*, 2003). In addition penicillin-binding proteins, which are septum-specific peptidoglycan synthetic enzymes, are required for cell division, illustrated by the induction of mycobacterial filamentation on treatment with β -lactam antibiotics (Mizuguchi *et al.*, 1985). It is conceivable that WhmD/WhiB2 regulate the expression of one or more of the above-mentioned genes, or other annotated cell-division-associated genes. Transcriptional studies to define the WhiB2 regulon in *M. tuberculosis* may help clarify the role of this protein in cytokinesis.

The continuous emergence of antibiotic resistance demands that novel classes of antibiotics continue to be developed. The division machinery of bacteria is an attractive target because it comprises several essential proteins that are conserved almost throughout the bacteria but are absent from humans. The discovery and development of small-molecule cell division inhibitors have primarily been focused on the

FtsZ protein, targeting either its intrinsic GTPase activity or specific protein-protein interaction (Jennings *et al.*, 2004; Margalit *et al.*, 2004; White *et al.*, 2002). These molecules have provided new insight into cytokinesis in bacteria and offer important reagents for further studies of the cell division machinery. Although significant progress has been made in our understanding of the physiology of *M. tuberculosis*, no definitive explanations exist for its extremely slow growth rate. Understanding the genetic mechanisms by which *M. tuberculosis* regulates the process of cell division is imperative to our overall comprehension of the process of latency. Of the 20 annotated cell division genes in the *M. tuberculosis* genome (Cole *et al.*, 1998), 10 are predicted to be essential and theoretically are targets for therapeutic intervention. The delineation of the mycobacterial cell division cycle could therefore play a vital role in stimulating the development of novel anti-tuberculosis agents.

Here we demonstrate that *M. tuberculosis* WhiB2 is functionally equivalent to its homologue WhmD in *M. smegmatis*. Our studies strongly suggest that *whiB2* is an essential gene and that its protein product is expected to regulate the expression of genes involved in mycobacterial cell division. WhiB2 therefore represents yet another cell division protein which could be a candidate for rational drug design.

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