whmD is an essential mycobacterial gene required for proper septation and cell division

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A study of potential mycobacterial regulatory genes led to the isolation of the *Mycobacterium smegmatis whmD* gene, which encodes a homologue of WhiB, a *Streptomyces coelicolor* protein required for sporulation. Unlike its *Streptomyces* homologue, WhmD is essential in *M. smegmatis*. The *whmD* gene could be disrupted only in the presence of a plasmid supplying *whmD* in trans. A plasmid that allowed chemically regulated expression of the WhmD protein was used to generate a conditional *whmD* mutant. On withdrawal of the inducer, the conditional *whmD* mutant exhibited irreversible, filamentous, branched growth with diminished septum formation and aberrant septal placement, whereas WhmD overexpression resulted in growth retardation and hyperseptation. Nucleic acid synthesis and levels of the essential cell division protein FtsZ were unaltered by WhmD deficiency. Together, these phenotypes indicate a role for WhmD in mycobacterial septum formation and cell division.

The slow growth rate of mycobacteria is significant to the pathogenesis and treatment of mycobacterial infections; yet little is known about cell division and its regulation in these bacteria. This slow growth rate necessitates protracted chemotherapeutic regimens for tuberculosis and leprosy, which in turn leads to poor patient compliance and the emergence of drug-resistant organisms.

Recently, much progress has been made toward an understanding of the temporal and spatial regulation of prokaryotic cell division (1–4). Doubling times for mycobacteria are significantly longer than those of better characterized model prokaryotes: 18–24 h in *Mycobacterium tuberculosis*, the cause of tuberculosis, 14 days in *Mycobacterium leprae*, the cause of leprosy, and 3 h in *Mycobacterium smegmatis,* a saprophyte. The basis for these prolonged cell division times is unknown, although the limited number of rRNA operons (one in *M. tuberculosis*, two in *M. smegmatis*, as compared with seven in *Escherichia coli*) and the metabolic costs of maintaining the complex cell wall that characterizes this genus are often cited as possible contributing factors (5–7).

Here we show that the *M. smegmatis whmD* gene is essential for cell division. *whmD* was identified in a search for homologues of *Streptomyces coelicolor* proteins known to function in postexponential phase adaptation. The WhmD protein is homologous to WhiB, an 87 aa *S. coelicolor* protein that is dispensable for normal vegetative growth but is required for the maturation of aerial hyphae during sporulation (8, 9). *S. coelicolor whiB* mutants fail to assemble FtsZ rings in their aerial hyphae, leading to the arrest of hyphal development before crosswall formation (10). WhiB is postulated to function as a transcription factor, although the exact mechanism by which it regulates spore formation is not yet established. Recently a family of *whiB*-like genes has been identified in *Streptomyces* and *Mycobacteria* (11), and functional assessments of some of these genes have been reported (12, 13). Our data show that WhmD, like WhiB, is important for the formation and maturation of the division septum.

Methods

Strains and Media. *M. smegmatis* mc²6 1-2c was grown in Middlebrook 7H9 liquid medium and on Middlebrook 7H10 agar. These media were supplemented with albumin dextrose complex (ADC; 5 g/liter BSA, 2 g/liter dextrose, 0.85 g/liter NaCl), 0.2% glycerol, and antibiotics as appropriate (50 μg/ml hygromycin, 25 μg/ml kanamycin, 30 μg/ml apramycin). Acetamide at either 0.2% or 0.02% was used to induce expression of *whmD* from pJG1012, and withdrawal of acetamide was achieved by washing cells in M7H9 twice. *E. coli* DH5α was used for all subcloning procedures and was grown in LB medium/agar.

Identification and Cloning of *M. smegmatis whmD*. The degenerate primers WB1AL (5′-GGGAATTCTGGCARSWVCRIGSICT-STG-3′) and WB4AR (5′-GCGCAAGCTTCGYTCSSWYTC-SSWSAGICCICCCCA-3′) were used to amplify a 200-bp fragment of the *M. smegmatis whmD* gene by PCR. This DNA fragment was used to probe a Lambda ZapII library of *Eco*RI-digested *M. smegmatis* genomic DNA obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Of approximately 4,000 plaques examined in the primary screen, one gave a positive signal after secondary screening. A phagemid with a 783-bp insert containing the *whmD* ORF was isolated and sequenced, and the size and origin of this fragment were confirmed by Southern blotting of *Eco*RI-digested *M. smegmatis* genomic DNA. The sequence of this DNA fragment has been entered into GenBank under accession number AF164439. To obtain a larger DNA fragment carrying the *whmD* locus, a pUC19-based plasmid library of 2.5- to 3.5-kb *Pml*I genomic DNA fragments was generated and probed with the 783-bp *Eco*RI restriction fragment. Two clones, pJGH1 and PJGI2, were isolated and found to contain the regions upstream and downstream of *whmD*, respectively.

Plasmid Constructs for *whmD* Gene Replacement. The targeting vector pJG1007 contains a 2.0-kb *Pvu*II fragment (containing approximately 1.9 kb of upstream sequences and the first 37 codons of *whmD*) and a 2.2-kb *Xba*I fragment (containing the *whmD* stop codon and downstream sequences) flanking the *hyg* gene from *Streptomyces hygroscopicus*. The hygromycin resistance cassette replaces the final 91 codons of the *whmD* coding region. The vector backbone of pJG1007 contains a kanamycin resistance cassette (*aph*) and *sacB* gene but does not contain a mycobacterial origin of replication and therefore can be maintained only by integration into the host chromosome (Fig. 1*A*).
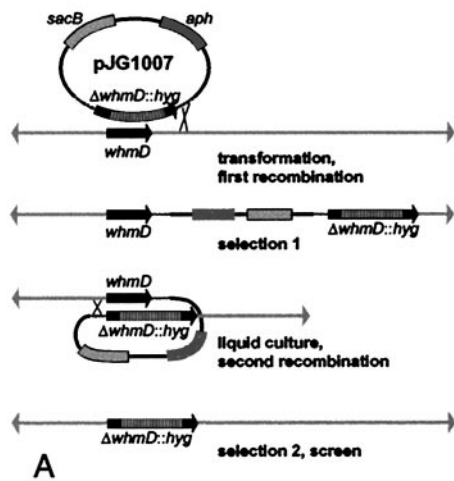
Fig. 1. (A) Two-step strategy for disruption of the *M. smegmatis whmD* gene. pJG1007 carries a partially deleted *whmD* gene with a cassette conferring Hy^R replacing the final 93 codons of *whmD*, a *sacB* marker conferring Suc^S, and an *aph* gene conferring Km^R. Selection 1 results in Km^R,Hy^R merodiploid integrants. Selection 2 (on sucrose) produces either the desired knockout (Suc^R, Hy^R, Km^S) as shown, the wild-type (Suc^R, Hy^S, Km^S), or *sacB* mutant merodiploids (Suc^R, Hy^R, Km^R). (B) Southern blot of total DNA from representative sucrose-resistant clones and their parental strains. A 2-kb *Bam*HI fragment containing *whmD* was used to probe *Bam*HI-digested genomic DNA run on a 0.8% agarose gel. The last three lanes show the three types of postsucrose selection isolates obtained when the trans-complementing plasmid pJG1010 was present. The final lane shows the loss of the wild-type 2-kb *Bam*HI fragment and the presence of the insertionally inactivated allele. A summary of the resistance phenotypes of analyzed clones is shown in Table 1, with each corresponding genotypic pattern as determined by Southern blotting. mc²6 E, Merodiploid strain obtained after selection 1. After sucrose selection, 10 clones from mc²6 E (pPE207) and 30 clones from mc²6 E (pJG1010) were scored phenotypically. The numbers at the bottom indicate the frequency of each phenotype.

The complementing plasmid pJG1010 consists of a 1.9-kb *Bam*HI-*Xba*I *whmD*-containing fragment cloned into the apramycin resistance-conferring shuttle vector pPE207 (14), kindly provided by Julian Davies (Univ. of British Columbia, Vancouver, BC, Canada). The acetamide-regulated complementing plasmid pJG1012 expresses *whmD* from the inducible acetamidase promoter of *M. smegmatis* (15). Its parental vector pJG1011, a pPE207 derivative, contains only the acetamidase regulatory region.

Replacement and Complementation of the *M. smegmatis whmD* Gene.
After electroporation into *M. smegmatis*, pJG1007 integrants were selected on hygromycin plus kanamycin. Southern blotting of *Pml*I-digested DNA isolated from *M. smegmatis* at this stage revealed that, in generating the merodiploid intermediate, homologous recombination between pJG1007 and the chromosomal *whmD* locus occurred with approximately equal efficiency in the upstream (18 of 37 clones) and downstream (19 of 37 clones) sequences flanking the hygromycin resistance (Hy^R) marker in pJG1007. After 24 h of growth in liquid culture in the absence of antibiotics, clones were subjected to a second round of selection on 10% sucrose with or without hygromycin, and sucrose-resistant clones were screened for kanamycin sensitivity. The complementing plasmids pJG1010 and pJG1012 were introduced into the merodiploid intermediate strain by electroporation. After sucrose selection, the presence of the *aph* gene, which cannot be scored phenotypically because of the apramycin resistance marker on the pPE207 derivatives, was scored by PCR.

Microscopy. Differential interference contrast (DIC) microscopy and fluorescence microscopy were used to examine *M. smegmatis* morphology. Live bacteria were stained for 5 min at 37°C with the nucleic acid binding dye SYTO 11 (Molecular Probes) at a concentration of 5 μg/ml in Middlebrook 7H9 medium. SYTO 11, which fluoresces when bound to either RNA or DNA, was chosen for our analyses because of its permeance relative to 4',6-diamidino-2-phenylindole (DAPI). Digital photography was performed by using a Photometrics (Tucson, AZ) PXL-1400 charge-coupled device (CCD) camera, and images were manipulated (background subtraction) by using the IPLAB program. Fluorescence images were artificially colored by using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Electron microscopy was performed on Zeiss EM10 or Phillips (Eindhoven, the Netherlands) CM120 instruments at the Johns Hopkins Medical Institutions Microscopy Facility. Three different fixation and staining protocols were used to maximize cell wall and nucleoid visualization and to orient the bacteria in a planar arrangement for sectioning. The first set of samples was fixed in 2% glutaraldehyde, 2% formaldehyde in Dulbecco's PBS (DPBS) (pH 7.4) plus 3 mM MgCl$_2$ for 1 h and then washed three times for 5 min each in 0.1 M cacodylate. Specimens were then microwaved for 5 min in 1% OsO$_4$ in 0.1 M cacodylate, washed three times for 5 min each in dH$_2$O, and treated with 2% uranyl acetate for 30 min before rinsing with 50% ethanol. Samples were dehydrated by sequential 10-min rinses in ethanol (50%, 70%, 90%, and 100%, three times each). After 1 h in SPURRS (a low viscosity resin) diluted 1:1 in ethanol, samples were transferred to complete SPURRS overnight. The following day, the resin was changed three times before baking at 60°C overnight. A second set of samples was subjected to the above protocol with minor modifications. In an attempt to obtain a more planar arrangement of the mycobacteria before sectioning, after dehydration the bacteria were embedded in eponate on a poly-L-lysine-coated coverslip. The third set of samples was handled as in Takede *et al.* (16).

Western and Northern Blot Analyses. Recombinant histidine-tagged WhmD was expressed in *E. coli* BL21(DE3) from pETD1, a derivative of pET15b containing the *whmD* ORF. The His-tagged WhmD protein was purified by using a nickel column (Invitrogen) and was used to immunize a New Zealand white (NZW) rabbit at Covance Laboratories (Denver, PA). WhmD antiserum was used at a 1:100 dilution in Western blots of *M. smegmatis* proteins that had been electrophoresed on a 12% tricine gel and transferred to nitrocellulose. Horseradish peroxidase-conjugated goat anti-rabbit IgG at a 1:3500 dilution and chemiluminescent substrate (Amersham) were used to detect the presence of WhmD. Detection of FtsZ by Western blot was
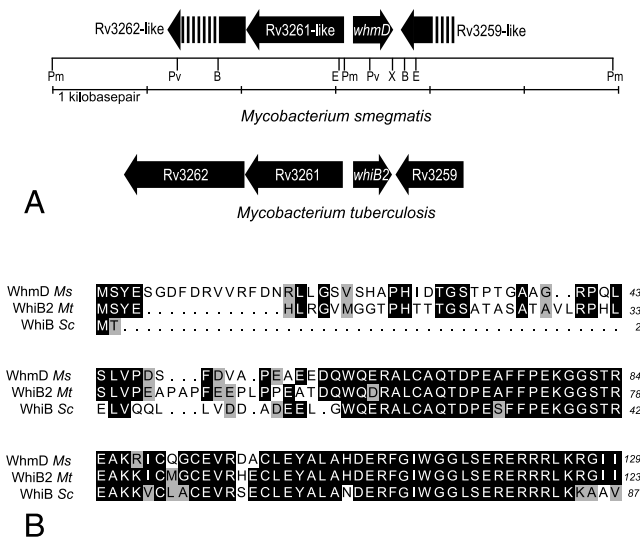
Fig. 2. (A) Map of the *M. smegmatis whmD* locus. The restriction sites in this region are shown as abbreviations (B = *Bam*HI, E = *Eco*RI, Pm = *Pml*I, Pv = *Pvu*II, and X = *Xba*I). The local arrangement of predicted genes is shown by arrows. The arrangement of genes in *M. tuberculosis* H37Rv is shown below to illustrate the similarity of the gene arrangement in the two species. (B) Alignment of the *M. smegmatis* (Ms) WhmD and *M. tuberculosis* (Mt) WhiB2 (WhmD) proteins with the WhiB protein of *S. coelicolor (Sc)*. Identities are shown in black and similarities in gray.

achieved by using the polyclonal rabbit antiserum UK25A, raised against the *E. coli* FtsZ protein, generously provided by Joe Lutkenhaus (Univ. of Kansas Medical Center, Kansas City, KS). *M. smegmatis* proteins were electrophoresed on a 10% SDS/PAGE gel before transfer, and UK25A serum was used at a 1:2000 dilution to detect the *M. smegmatis* FtsZ protein.

Results

Identification of the *M. smegmatis whmD* Gene. Southern blot surveys suggested the existence of a mycobacterial gene closely related to *whiB* of *S. coelicolor* (17). A partial *M. smegmatis* ORF was amplified by degenerate PCR using primers based on the sequence of the *S. coelicolor whiB* gene, and a full-length clone was subsequently isolated from a genomic library of *M. smegmatis* using the PCR product as a probe. We designated this gene *whmD*, because it was the fourth mycobacterial *whiB* homolog we had identified (11). A highly similar *M. tuberculosis* gene later appeared in the published genome sequence of the H37Rv strain and was annotated as *whiB2* (18). The genomic context of the *M. tuberculosis* and *M. smegmatis whmD* genes is conserved (Fig. 2*A*). Additionally, whereas the C termini of the 129-aa *M. smegmatis* WhmD (WhmD$_{Ms}$) protein and the 123-aa WhmD$_{Mt}$ (WhiB2) are virtually identical to the 87-aa *S. coelicolor* WhiB, the WhmD proteins contain a unique 38- to 48-aa N-terminal extension not found in WhiB (Fig. 2*B*). Although the *M. tuberculosis whiB2* gene is annotated as an 89 codon ORF based on homology to *whiB*, the use of the more upstream start codon is supported by both the relative mobility of the WhmD protein seen in immunoblots (15–16 kDa) and the presence of a likely Shine-Dalgarno sequence centered 10 bp upstream of the proximal start codon. A total of seven WhiB-homologues are encoded in the *M. tuberculosis* genome and at least six are present in *S. coelicolor*; however, the gene family has not been identified in bacteria outside the order Actinomycetales (11).

***whmD* Is an Essential Gene.** We attempted to disrupt the *M. smegmatis whmD* locus by a two-step gene replacement strategy by using a suicide vector in which a hygromycin-resistance

cassette replaced the final 91 codons of the *whmD* ORF (Fig. 1*A*). The vector also contained the counterselectable *sacB* gene and the *aph* gene, which confer sucrose sensitivity (Suc^S) (19, 20) and kanamycin resistance (Km^R), respectively. After electroporation, numerous Hy^R, Km^R merodiploid clones were obtained, and the integration of the targeting vector at the *whmD* locus was confirmed by Southern blotting. These merodiploids were subjected to the second-step selection on medium containing sucrose. Clones capable of growth on sucrose were anticipated in three categories: Δ*whmD*::*hyg* (Hy^R, Suc^R, and Km^S), wild-type (Hy^S, Suc^R, and Km^S), or *sacB* mutant merodiploids (Hy^R, Suc^R, and Km^R). Unexpectedly, no Hy^R, Suc^R, and Km^S colonies (i.e., Δ*whmD*::*hyg* recombinants) were obtained after multiple attempts. As shown in Table 1, a detailed phenotypic evaluation of 742 Suc^R colonies obtained by second-step selection at either 30°C or 37°C failed to reveal a single *whmD*-disrupted mutant. However, Suc^R clones resulting from recombination on the same flank as the original integration, restoring an intact *whmD* locus, were readily obtained when hygromycin selection was omitted. Because the initial integration of the plasmid occurred with equal frequency on either flank, the failure to obtain knockouts could not be explained by recombinational bias toward one flank of the *whmD* gene. Instead, these results strongly suggested that disruption of *whmD* is deleterious for bacterial survival.

To evaluate whether *whmD* is in fact an essential gene, we introduced a *whmD*-containing plasmid conferring apramycin resistance (Am^R) into the Hy^R,Km^R merodiploid before sucrose counterselection. The complemented merodiploid strain gave rise to 2 of 30 (6.7%) Hy^S, Km^S wild-type revertants, 26 of 30 (87%) Hy^R, Km^R *sacB* mutants, and 2 of 30 (6.7%) Hy^R, Km^S chromosomal *whmD*-disrupted mutants. Although the majority of Suc^R clones arose because of *sacB* mutation, plasmid excision events leading to the loss of the Km^R marker occurred at an equal frequency on either flank. Thus, the presence of an episomal *whmD* gene permitted the disruption of the chromosomal *whmD* locus. Transformation with the parental vector pPE207 had no effect on our ability to disrupt the chromosomal *whmD* gene. Because the complementing plasmid contained no ORFs 3′ of *whmD*, successful disruption of the chromosomal allele only in the presence of extrachromosomal complementation indicates that *whmD*, itself, rather than a downstream gene, is essential in *M. smegmatis*.

Conditional Complementation Reveals a Role for WhmD in Septation and Cell Division. To assess the phenotypic consequences of *whmD* loss, we developed a strain in which the chromosomal *whmD* disruption could be conditionally complemented by *whmD* expression from an inducible promoter. We replaced the native *whmD* promoter with an acetamide-inducible promoter and regulatory gene cassette from the *M. smegmatis* acetamidase gene locus. Recently, this 3-kb *M. smegmatis* DNA cassette, which contains several putative accessory genes and a strong promoter (P$_{ace}$) preceding the highly inducible acetamidase gene, has been shown to confer acetamide inducibility on downstream genes (15). After a plasmid, pJG1012, harboring the P$_{ace}$::*whmD* allele was introduced into a Hy^R, Km^R *whmD* merodiploid strain, sucrose selection in the presence of 0.2% acetamide yielded a Hy^R,Km^S strain, designated 628-53, that on Southern blot analysis contained the desired chromosomal Δ*whmD* mutation. Immunoblots of lysates of *M. smegmatis* 628-53 showed that, in the presence of acetamide, WhmD was produced at levels greater than those seen in wild-type *M. smegmatis*, but WhmD levels declined rapidly after acetamide withdrawal (see Fig. 5). The addition of 0.2% acetamide to wild-type *M. smegmatis* did not alter WhmD expression levels.

When strain 628-53 was transferred from medium containing glycerol and acetamide to medium containing only glycerol, the

**Table 1. Results of second-step selection on 10% sucrose**

Temperature	Selection medium*	No. of colonies (%)			
		Hy^{R}Kan^S ΔwhmD	Hy^{S}Kan^S wt whmD	Hy^{R}Kan^R sacB mutant	Total
37°C	M7H10 + 10% sucrose	0 (0%)	114 (70%)	50 (30%)	164
	M7H10 + 10% sucrose, hygromycin B	0 (0%)	0 (0%)	275 (100%)	275
30°C	M7H10 + 10% sucrose	0 (0%)	95 (40%)	144 (60%)	239
	M7H10 + 10% sucrose, hygromycin B	0 (0%)	0 (0%)	64 (100%)	64

*M7H10, Middlebrook 7H10 medium supplemented with albumin dextrose complex (ADC).

WhmD-underexpressing cells became defective in cell division, forming long, branched filaments over the course of 6 to 12 h (6 h equates to slightly more than two typical generations) (Figs. 3 and 4). During this time, the culture continued to accumulate biomass as assessed by turbidity measurements, and increasing filamentation resulted in macroscopic aggregation of the bacilli. Qualitative analyses showed that bacterial length increased rapidly after acetamide withdrawal, suggesting that the effect of *whmD* loss was immediate. Addition of acetamide to the culture medium, after filamentation had become obvious, did not lead to restoration of binary fission. The WhmD-deficient filaments retained their acid-fast staining properties.

We studied the filaments for the presence of septa by examining thin sections of fixed samples by transmission electron microscopy (Fig. 3) (16). Although septa were observed in strain 628-53 grown in both acetamide-free and acetamide-containing media, the frequency, positioning, and character of the septa were altered when WhmD levels were depleted by acetamide withdrawal. Whereas wild-type *M. smegmatis* grown in the presence of acetamide were limited to a single, medially placed
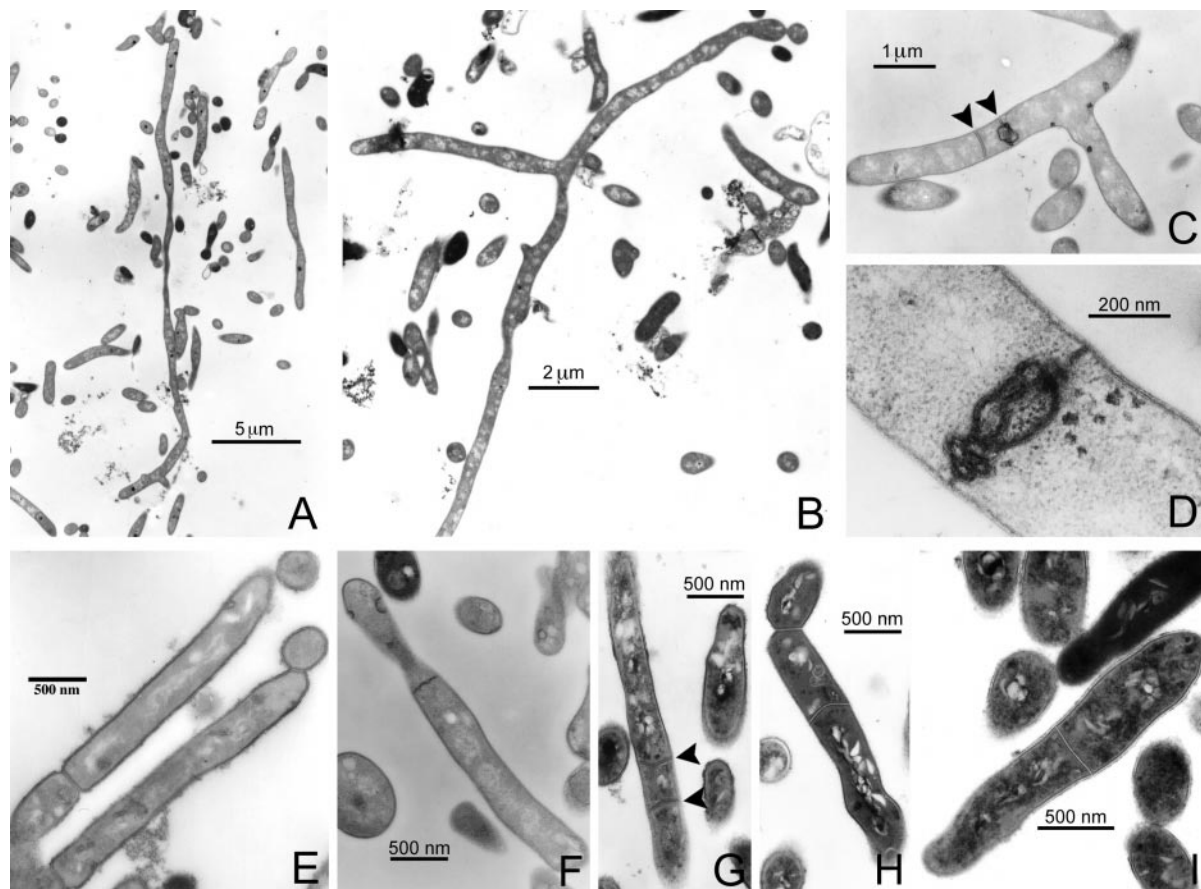
Fig. 3. (*A–D*) Transmission electron micrographs of ultrathin sections of *M. smegmatis* 628-53, which carries the plasmid pJG1012 (P$_{ace}$::*whmD*), allowing for the acetamide-dependent expression of WhmD. After growth for 13 h in the absence of acetamide, bacteria became filamentous, as can be seen in *A* and *B*. The longest bacterium in *A* is at least 26 μm in length, and no evidence of septa can be detected throughout its length. In *B*, a branched, aseptate bacterium is shown. The length of this bacterium from the upper right corner to the bottom center of the panel is 16 μm. (*C* and *D*) Defective septal structures seen in the WhmD-underexpressing mutant; (*C*) closely spaced septa (arrows) present in sections of single filaments. (*E* and *F*) The complemented mutant strain grown in 0.2% acetamide. Although the morphology of septa appears to be occasionally abnormal, as seen in *F*, these bacteria are capable of septum maturation and fission. (*G–I*) *M. smegmatis*-carrying plasmids pJG1012 (P$_{ace}$::*whmD*; *G* and *H*) or PJG1011 (P$_{ace}$, empty vector; *I*). Bacteria in *G–I* were grown 0.2% acetamide and were fixed and stained by using tannic acid. Noteworthy are the multiple septa within a single bacterium (*G*, arrows) and asymmetric positioning of septa close to previous division sites (*H*). No unusual septation was seen in bacteria carrying the control plasmid pJG1011 (*I*).
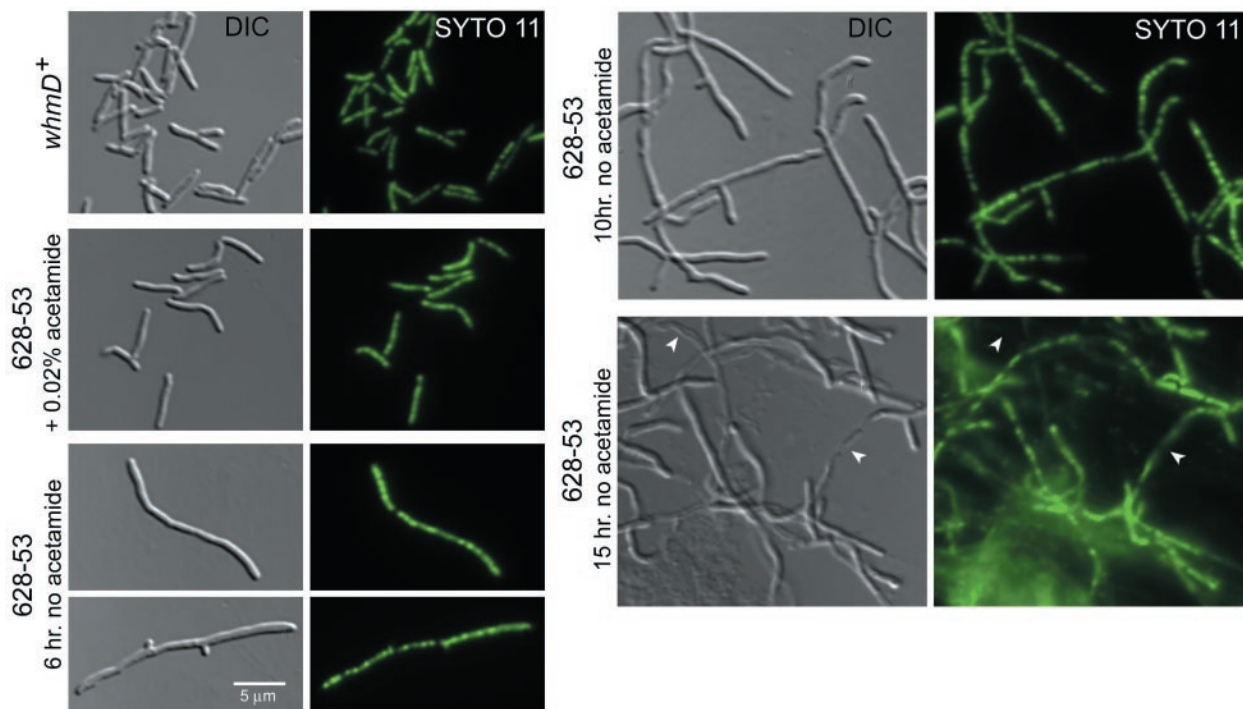
Fig. 4. DIC and fluorescence microscopy analysis. The nucleic acid stain SYTO 11 was used to localize the nucleoids in wild-type and *whmD* mutant *M. smegmatis* grown in M7H9 with and without 0.2% acetamide. Parallel digital micrographs of the same field were captured by using DIC (*Left*) or fluorescence (*Right*) microscopy (×1000). After two washes in acetamide-free M7H9, *M. smegmatis* 628-53 was grown at 37°C in acetamide-free M7H9 (with 0.2% glycerol as a carbon source). The formation of filaments and some branching is obvious by 6 h (doubling time of *M. smegmatis* is 2.5 to 3 h). By 10 h, bacteria have become highly branched and filamentous, and larger, more obvious gaps in the nucleic acid staining pattern are visible. Significant lysis is seen by 15 h (arrows). Wild-type *M. smegmatis* mc²6 1-2c (*whmD*+) and the complemented mutant (628-53 + 0.2% acetamide) are shown for comparison.

septum, the filamentous WhmD-deficient organisms often had multiple septa distributed unevenly throughout their length. In the absence of acetamide, the placement of completed septa was not medial; septa were commonly observed near branch points and the tips of filaments. Some WhmD-deficient filaments were completely devoid of septa; one such filament, from a culture grown for 13 h without acetamide, was 26 microns long. When septa were present in the mutant, their maturation appeared to be arrested because no evidence of cytokinesis was seen in mutant cells. In the presence of acetamide, *M. smegmatis* 628-53 was competent for cytokinesis (Fig. 3*E*), although the structures of some septa observed by transmission electron microscopy appeared to be abnormal, as illustrated in Fig. 3*F*.

Having observed that WhmD withdrawal reduced the frequency of septum formation, we investigated the effect of WhmD overexpression. Wild-type *M. smegmatis*, harboring the P*ace*::*whmD* fusion plasmid (pJG1012) grown in the presence of acetamide, produced excess WhmD relative to similarly grown *M. smegmatis* carrying a plasmid bearing only P*ace* (pJG1011). On M7H10 agar, the *M. smegmatis* colony size was greatly reduced when *whmD* expression was induced by the presence of acetamide in the medium, but the WhmD-overexpressing strain showed only slightly slowed growth kinetics in liquid medium. Although liquid-grown, acetamide-induced *M. smegmatis* carrying pJG1012 appeared normal by DIC microscopy, we observed several examples of multiply septate bacteria when these cultures were examined by transmission electron microscopy (Fig. 3*G*). These observations suggest that WhmD levels affect the localization or efficiency of septum formation. Interestingly, whereas excess WhmD in a wild-type background led to misplaced septa, greater than wild-type levels of WhmD were required in trans to prevent filamentation in a Δ*whmD*::*hyg* background.

We evaluated the state of the nucleoids in the filamentous,

WhmD-deficient *M. smegmatis* 628-53 by staining with SYTO 11, a highly permeant dye that fluoresces when bound to nucleic acids (Fig. 4). SYTO 11-stained filaments fluoresced in a beaded pattern throughout their length, suggesting that they contained multiple chromosomes and that nucleic acid synthesis had continued after WhmD withdrawal. After 10 h of growth in the absence of acetamide, larger gaps between nucleoids could be observed, and a significant amount of lysis was seen. By 15 h, we observed many instances of partially lysed filaments by DIC microscopy. These partial ghosts failed to stain with SYTO 11 in the lysed segments of their length but continued to stain in their unlysed segments, indicating that the septa present in these filaments can completely separate adjacent cytoplasmic compartments. These experiments suggest that the WhmD-deficient filaments are polyploid with respect to DNA content.

Withdrawal of WhmD Does Not Affect FtsZ Accumulation. The polymerization of FtsZ in a ring at midcell is necessary for initiation of septum assembly. To determine whether WhmD deficiency reduced the expression of FtsZ, we performed Western blots of *M. smegmatis* 628-53 underexpressing WhmD during various stages of filamentation (Fig. 5). Despite the extensive filamentation by 6 h, FtsZ levels remained constant from 1 to 24 h whereas WhmD levels declined rapidly, reaching minimal levels after 3 h. Hence, the septal defects we observed after WhmD withdrawal are not due to insufficient levels of FtsZ.

Discussion

The cell division defects associated with WhmD deficiency provide an explanation for the essential nature of the *whmD* gene. Although loss of *whmD* has more severe consequences for *M. smegmatis* than does loss of *whiB* for *S. coelicolor*, the two mutant phenotypes are related. Both WhiB and WhmD appear
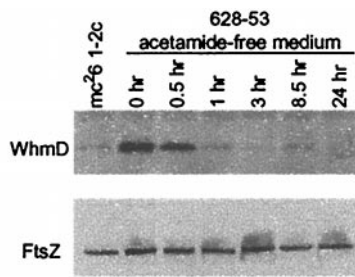
Fig. 5. WhmD withdrawal does not influence FtsZ accumulation. Western blots to detect WhmD and FtsZ in lysates of *M. smegmatis* 628-53 at various times after transfer of the bacteria to acetamide free medium. Lysates containing matched amounts of protein were separated on a 12% tricine gel for detection of the 14.4-kDa WhmD protein, or on a 10% SDS/PAGE gel for detection of the ≈39-kDa FtsZ protein.

to be important for septum formation and fragmentation, WhiB governing postexponential phase spore septum formation and WhmD binary fission. Whereas *whiB* expression is tightly linked to growth phase (21), both *whmD* mRNA and WhmD protein are present throughout exponential growth and into early stationary phase but are undetectable in 5-day-old cultures (data not shown). This expression pattern is consistent with a requirement for WhmD during periods of active cell division. The fact that WhiB is dispensable in *S. coelicolor* likely reflects the mycelial nature of this organism; Streptomycetes are unique in their ability to remain viable after deletion of the usually essential cell division genes *ftsZ* and *ftsQ* (10, 22, 23). The extensive and immediate filamentation we observed in a conditional *whmD* mutant, in conjunction with an apparent defect in septum formation and positioning, suggests a role for WhmD 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*.

Several other proteins closely related to WhmD and WhiB are present in *M. tuberculosis*, *S. coelicolor*, and other high-GC Gram-positive bacteria. These proteins are apparently incapable of functionally substituting for WhmD, because loss of *whmD* alone is lethal. A homologue of *whmD*, *whmB* (also described as *whiB3*), is present in *M. smegmatis*, *M. tuberculosis*, and *M. leprae*. Although this gene is nonessential in *M. smegmatis* (13), the *S. coelicolor* orthologue of WhmB, WhiD, has been shown to be essential for spore formation (12).

It should be noted that the filamentous, largely aseptate morphology of the WhmD-deficient *M. smegmatis* 628-53 is similar to that seen when *Mycobacterium avium* is treated with low levels of the β-lactam antibiotic ampicillin (24). Several penicillin-binding proteins have been shown to be required for cell division; examples include the *E. coli* FtsI (PBP3) (25) and its *Bacillus subtilis* counterpart, PBP2B (26), which are septum-specific peptidoglycan synthetic enzymes. *ponA* mutants of *B. subtilis*, which are defective in PBP1 function, also show sporadic division defects (27). Along these lines, the septal defects of 628-53 may be due to a WhmD requirement for function of one or more cell division-associated murein synthetic enzymes. A better understanding of WhmD function may reveal additional components of the actinomycete cell division machinery and provide new potential targets for chemotherapeutic intervention against mycobacterial diseases.

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