Physiological consequences associated with overproduction of *Mycobacterium tuberculosis* FtsZ in mycobacterial hosts

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The *ftsZ* gene of *Mycobacterium tuberculosis* H37Rv has been characterized as the first step in determining the molecular events involved in the cell division process in mycobacteria. Western analysis revealed that intracellular levels of FtsZ are growth phase dependent in both *M. tuberculosis* and *Mycobacterium smegmatis*. Unregulated expression of *M. tuberculosis* *ftsZ* from constitutive *hsp60* and *dnaA* promoters in *M. tuberculosis* hosts resulted in lethality whereas expression from only the *hsp60* promoter was toxic in *M. smegmatis* hosts. Expression of *ftsZ* from the *dnaA* promoter in *M. smegmatis* resulted in ~ sixfold overproduction and the merodiploids exhibited slow growth, an increased tendency to clump and filament, and in some cases produced buds and branches. Many of the cells also contained abnormal and multiple septa. Expression of *ftsZ* from the chemically inducible acetamidase promoter in *M. smegmatis* hosts resulted in ~ 22-fold overproduction of FtsZ and produced filamentous cells, many of which lacked any visible septa. Visualization of the *M. tuberculosis* FtsZ tagged with green fluorescent protein in *M. smegmatis* by fluorescence microscopy revealed multiple fluorescent FtsZ foci, suggesting that steps subsequent to the formation of organized FtsZ structures but prior to septum formation are blocked in FtsZ-overproducing cells. Together these results suggest that the intracellular concentration of FtsZ protein is critical for productive septum formation in mycobacteria.

Keywords: Cell division, filamentation, *Mycobacterium smegmatis*, FtsZ–GFP, Z ring

INTRODUCTION

In recent years there have been major advances in our understanding of the molecular aspects of the cell division process in *Escherichia coli*. These studies have suggested that cell division is a complex multi-protein process that requires the assembly of several protein components in a precisely ordered manner and that FtsZ, the initiator of the cell division process, is critical for this assembly (Lutkenhaus & Addinall, 1997; Margolin, 2000). FtsZ, a structural homologue of tubulin (Lowe & Amos, 1998), is the most highly conserved of the bacterial cell division proteins. Homologues of FtsZ have been found in all eubacterial species that have been examined with the exception of *Aeropyrum pernix*, *Ureaplasma urealyticum* and Chlamydiae (recently reviewed by Margolin, 2000). FtsZ has been shown to be present in archaea and plant organelles (see Margolin, 2000). It is believed that the *ftsZ* gene product acts at an early step in the cell division process and that its concentration is rate limiting for cell division (Bi & Lutkenhaus, 1990; Ward & Lutkenhaus, 1985). Further, FtsZ has been shown to localize to the site of division septa in the form of specialized structures called Z rings (Bi & Lutkenhaus, 1991). Conditional mutants of *ftsZ* in *E. coli* are deficient in cell division and under nonpermissive conditions produce long filamentous cells (termed filamentation). Although these mutants complete the replication cycle and segregate the nucleoids, they lack any signs of cellular constrictions (reviewed by Lutkenhaus & Addinall, 1997). Some of the proteins involved in the cell division process in *E. coli*, such as FtsA, FtsN, FtsI, FtsQ etc., have also been shown to localize to the Z ring, suggesting that formation of the Z ring...

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Abbreviation: GFP, green fluorescent protein.
ring is the defining cytoskeletal structure that marks the beginning of the cell division process both spatially and temporally (Margolin, 2000; Rothfield et al., 1999). Besides E. coli, ftsZ has been characterized from only a handful of bacteria, e.g. Bacillus subtilis, Caulobacter crescentus, Rhizobium meliloti and Streptomyces sp. (Latch & Margolin, 1997; Ma et al., 1997; McCormick et al., 1994; Quadrokus et al., 2001; Sackett et al., 1998; Schwedock et al., 1997; Van Wezel et al., 2000; Wang & Luktenhaus, 1993). Many of the studies with ftsZ from other organisms have focused on cloning and expression in E. coli hosts. These studies have consistently shown that overexpression of heterologous ftsZ in E. coli hosts results in filamentation (Beall et al., 1988; Beall & Luktenhaus, 1991; Gaikwad et al., 2000; Salimnia et al., 2000; Yaoi et al., 2000).

Tuberculosis is an important infectious disease with over one billion people subclinically infected and three million deaths reported each year. The causative agent, Mycobacterium tuberculosis, is a slow growing bacterium with an approximate doubling time of 24 h. Although cell division and consequent multiplication of the bacterium are important steps in establishing infections, little is known about the molecular genetic aspects of the M. tuberculosis cell duplication process, specifically its initiation and regulation. This is important considering the slow growth rate of M. tuberculosis relative to other bacteria as well as its ability to shift between an active, multiplicative (infectious) state and a latent or quiescent state during growth in vivo. In the latent state, the tubercle bacillus after initial infection is believed to persist in a metabolically active but non-growing state which can reactivate and initiate disease when the immune system is compromised (reviewed by Bloom & Mckinney, 1999). In many bacteria, the ftsZ gene region includes ftsQ and ftsA genes. The FtsA protein is believed to serve as a molecular bridge between the periplasmic cell division proteins and FtsZ. Neither the ftsZ region nor other regions of the M. tuberculosis genome contain obvious ftsA homologues. The M. tuberculosis cell division machinery does however, appear to include a few novel putative cell division genes which are not present in E. coli, e.g. Rv0435c and Rv2115c (Cole et al., 1998).

The genus Mycobacterium also includes rapid growers with a doubling time of 2–3 h, e.g. Mycobacterium smegmatis. Currently the project to decipher the genome sequence of M. smegmatis is nearly complete (http://www.tigr.org/tdbmdb/mdbinprogress.html) and our analysis of the available sequence data indicated that its genome contains homologues of all the annotated cell division genes of M. tuberculosis. Furthermore, the M. smegmatis FtsZ protein sequence is 92% identical to that of its M. tuberculosis counterpart, with the majority of the differences present in the C terminus. Considering the similar composition of the cell division machineries of M. smegmatis and M. tuberculosis, one would expect that the cell division process, if not its regulation, to be similar in the two organisms. By the same token, it would appear that at least some aspects of the cell division process in mycobacteria and possibly its regulation will prove to be different from that of E. coli.

Here we report on the characterization of M. tuberculosis ftsZ in mycobacterial hosts. Our results indicate that the intracellular levels of M. tuberculosis FtsZ are growth-phase dependent and that elevated intracellular levels of FtsZ affect growth and interfere with cell division. This resulted in cells with abnormal and multiple septa and in some cases caused filamentation followed by cell lysis. Finally, as a first step towards the localization of M. tuberculosis FtsZ and its associated structures during the cell division process, we tagged FtsZ with green fluorescent protein (GFP) and visualized the fusion protein by fluorescence microscopy. Together our results indicate that FtsZ protein plays a critical role in the cell division process in mycobacteria.

METHODS

Bacterial strains, growth media and transformation conditions. The following bacterial strains were used: Escherichia coli Top10 (Invitrogen), Mycobacterium smegmatis mc²155 (Jacobs et al., 1991) and Mycobacterium tuberculosis H37Rv (Qin et al., 1999). E. coli was grown in Luria–Bertani (LB) media and mycobacterial strains were grown in Middlebrook 7H9 broth supplemented with oleic acid albumin dextrose catalase (OADC) as described by Qin et al. (1999). The E. coli transformants were selected on LB agar plates containing ampicillin or kanamycin (50 µg ml⁻¹ each) and the mycobacterial transformants were selected on Middlebrook 7H10 agar plates containing OADC and kanamycin (25 µg ml⁻¹) (Madiraju et al., 1999; Qin et al., 1999). Individual mycobacterial transformants were always colony purified, their plasmid DNA was recovered from E. coli following the bead-beating protocol (Madiraju et al., 1999) and the presence of cloned insert was confirmed by restriction digestion. Acetamide at a final concentration of 1% (w/v) in broth and 2% on solid agar media was used to induce FrsZ overproduction in M. smegmatis and M. tuberculosis hosts although concentrations of acetamide from 0.4 to 2% were found to be equally effective.

For growth phase experiments, approximately 20 ml mycobacterial cultures were grown in sealed 75 ml tissue culture flasks at 37 °C either in a slanting position (M. tuberculosis) or with shaking (M. smegmatis) for various times. The growth periods analysed included exponential, late-exponential, stationary and late-stationary phases. Under these culture conditions, M. tuberculosis cultures grew to a maximum OD₉₀₀ of 1.95; continued incubation for another 16 days did not result in any further increase in the optical density. The M. tuberculosis cultures harvested at OD₉₀₀ of 1.95 were referred to as stationary-phase cultures and those harvested after an additional 16 days growth were referred to as late-stationary-phase cultures. The M. tuberculosis cultures harvested at OD₉₀₀ of 0.96 were referred to as exponential- and late-exponential-phase cultures, respectively. Parallel experiments revealed that the oxygen content of stationary-phase cultures was totally depleted as indicated by the disappearance of methylene blue indicator dye (data not shown). The growth states of late-stationary-phase cultures described above are essentially similar to those used to attain the nonreplicating persistent state under oxygen depletion conditions (Wayne & Hayes, 1996). The M. smegmatis cultures harvested at OD₉₀₀ of 0.59, 0.98, 1.93 and 2.62 were
referred to as exponential-, late-exponential-, stationary- and late-stationary-phase cultures, respectively.

**Cloning of M. tuberculosis*ftsZ* in mycobacterial expression vectors.** Standard molecular biology protocols were used (Sambrook *et al*., 1989). Unless otherwise mentioned, all PCR products were first cloned into a TA vector (pGEM-T Easy; Promega), then released by digestion with appropriate restriction enzymes before cloning into expression vectors (see below). To facilitate subcloning into expression vectors, restriction enzyme recognition sites (underlined, see below) were incorporated in the sequence of the oligonucleotides.

**Cloning downstream of the acetamidase (ace) promoter.** The *M. tuberculosis ftsZ* coding region was amplified from the genomic DNA of *M. tuberculosis* H37Rv using the oligonucleotide primers MVM174 (5′-CGGATCCATGCCCGACCCCGCC-3′) and MVM125 (5′-CGGGATCTACGCCGGCGCATG-3′). The *ftsZ* coding region was cloned into the BamHI site of pJAM2, an *E. coli*–mycobacteria shuttle vector containing the 1.5 kb ace promoter region (Triccas *et al*., 1998) to create an in-frame fusion with the first six codons of the *amiC* gene. This construct (pSAR16) was referred to as *ace–ftsZ*.

**Cloning downstream of the hsp60 promoter.** The *ftsZ* coding region was amplified using the oligonucleotide primers MVMM24 and MVMM125 and cloned into the BamHI site of pMV261, an *E. coli*–mycobacteria shuttle vector containing the hsp60 promoter (Stover *et al*., 1991) to create an in-frame fusion with the first six codons of hsp60. The sequence of MVMM24 is essentially identical to that of MVM174 except that it contains the nucleotide ‘A’ immediately after the BamHI restriction site and before the ATG start codon. This construct (pFR12) was referred to as hsp60–*ftsZ*.

**Cloning downstream of the dnaA promoter.** This was done in two steps. First, the *ftsZ* coding region was amplified using the oligonucleotide primers MVMM24 and MVMM139 (5′-GGGAAGCCTTTGAGGCGCATGAGGCGGCCG-3′) and cloned into the BamHI/HindIII sites of pMV206, a promoterless *E. coli*–mycobacteria shuttle vector (Stover *et al*., 1991) to create pFR22. Next, a 587 bp fragment containing the *Mycobacterium avium* dnaA promoter region was amplified from pMQ436 (Madiraju *et al*., 1999) using the primers MVM141 (5′-CTAGTATACGCCGATCTGCCGACCGA-3′) and MVM142 (5′-GGTCATTGATCCTGGCGATCCGACCGG-3′), digested with BamHI/XbaI and ligated with pFR22 previously digested with the same enzymes to create an in-frame fusion with the *M. avium* dnaA promoter and the first three codons of *dnaA* gene. This construct (pFR32) was referred to as dnaA–*ftsZ*.

**Construction of the ftsZ–gfp fusion expressing plasmid.** The *ftsZ* gene was amplified by PCR from pSAR16 using oligonucleotides MVM174 (see above) and MVM185 (5′-GCTCTACGAGGGCGCATGAGGCGGCCG-3′) and cloned into the BamHI/XbaI sites of pJAM2 to create pJFR10. The *ftsZ* coding region in this construct lacks the stop codon and is expressed from the ace promoter. Next, a bright allele of the gfp gene (green fluorescent protein) was amplified from pFV25 (Cormack *et al*., 1997) using MVM188 (5′-GCTCTAGAAACACAAACCTGCAGATGATGAAAGGAGG-3′) and MVM189 (5′-GCTCTAGATTTGATATCATGCC-3′) oligonucleotide primers and cloned into the XbaI site of pJFR10 to produce pJFR11. The recombinant protein encoded by pJFR11 has the sequence SRNQNQ separating the FtsZ and GFP proteins. The gfp gene in pJFR11 lacks its own stop codon but contains one after the his-tag linker sequence, which adds the amino acids SRHHHHHH to the C terminus of GFP. A control plasmid, pJFR14, expressing gfp from the ace promoter was constructed by amplifying the gfp coding region from pJFR11 using the oligonucleotides MVM186 (5′-CGGGATCCATGAGTAAAGGAGAAG-3′) and MVM187 (5′-CGGGATCCCTGAGACTTGTATTTGGTTATATGTCATC-3′) and cloning the PCR fragment into the BamHI site of pJAM2.

**Viability experiments.** Appropriate dilutions of actively growing cultures of the *M. smegmatis* acep–*ftsZ* merodiploid strain were plated on Middlebrook agar plates supplemented with OADC and kanamycin, both in the presence and absence of 2% acetamide. The plates were incubated at 37 °C and the colonies from both series of plates were counted. Representative colonies from the acetamide plates were propagated, and plasmid DNA was recovered and analysed for the presence of the *ftsZ* insert. In some experiments, the ability of these cells to overproduce FtsZ protein following induction with acetamide was examined by Western blot analysis using anti-FtsZ antibodies (see below).

**Preparation of mycobacterial cell lysates and Western blotting.** Mycobacterial cell lysates were prepared by the bead-beating protocol using 0.1 mm zirconia glass beads as described by Yamamoto *et al*., 2001). Known amounts of cell lysates based on the equivalent cell number or protein concentration were resolved in SDS–polyacrylamide gels (Laemmli, 1970) and transferred to Protran nitrocellulose membranes (Schleicher and Schuell) as described by Yamamoto *et al*., 2001). Nitrocellulose blots were incubated with 200-fold diluted affinity-purified *M. tuberculosis* FtsZ antibodies and processed using the Amersham Pharmacia ECF chemifluorescence kit and protocol. Known amounts of recombinant *M. tuberculosis* FtsZ protein (A. A. L. Atkinson, S. A. Rutherford, M. V. V. S. Madiraju & M. Rajagopalan, unpublished) were used as standards and the FtsZ protein was visualized using a Bio-Rad Molecular Imager and quantitated using the Quantity One program (Bio-Rad). The amount of *M. tuberculosis* FtsZ protein was calculated and presented as a percentage of the total protein. In addition, the number of c.f.u. (ml culture suspension)1 at appropriate OD600 was determined and used to calculate the number of FtsZ molecules per live cell. While the latter method is accurate for the determination of FtsZ levels in actively growing cells, it could overestimate FtsZ levels in late-stationary-phase cells as some of these cells may be non-viable. We found that under the experimental conditions described the total protein per cell decreased during stationary phase. Often the Western blot analysis revealed the presence of two FtsZ bands—one band of ~40 kDa corresponded to the size of intact FtsZ protein and second to a smaller polyepitde of ~38 kDa. This was seen also in lysates prepared from wild-type *M. tuberculosis* cells. Presumably, the fast migrating polyepitde resulted from proteolytic degradation of the full-length FtsZ during cell-free lysate preparation. When present, both bands were included for the determination of FtsZ levels.

**Microscopy.** Mycobacterial cells were resuspended in buffer A [10 mM Tris/HCl, pH 7.5, 10 mM MgCl2, 0.02% (v/v) Tween 80], sonicated for 90 s in a Branson waterbath sonicator to break clumps and examined by conventional microscopy on either an Olympus Vanox-T AH-2 microscope with 100× DIC oil-immersion objective with a numerical aperture of 1.3 or a Zeiss photomicroscope with a 40× phase-contrast objective. FtsZ–GFP fluorescence images were acquired using a 100 W mercury lamp and the Piston GFP filter set (Chroma 41025 Piston). Some of the images were acquired on a Nikon FX-A microscope with a 60× oil immersion objective (numerical aperture 1.4) using a 100 W mercury lamp.
lamp and the standard FITC filter set. Mycobacterial cells were permeabilized by exposing to 2% (v/v) toluene for 2 min prior to staining for DNA either with a combination of ethidium bromide (40 μg ml⁻¹) and mithramycin (180 μg ml⁻¹) for 30 min on ice or with DAPI for 3 min at room temperature. In our hands, the mithramycin/ethidium bromide combination worked well for co-visualisation of DNA and FtsZ–GFP structures. Mithramycin is a DNA-specific dye with a low fluorescence yield and hence had to be used in combination with ethidium bromide (which itself has a negligible absorption at the excitation wavelength used) to obtain a higher fluorescence yield. Thus, ethidium bromide excitation is primarily by energy transfer from a mithramycin molecule bound nearby (<5 nm away) and this yields three to four times more fluorescence than can be obtained with mithramycin alone (Steen et al., 1986). Slides were examined using the Olympus 1B filter set (excitation wavelength 495 nm, dichroic mirror 505 nm and a broad bandpass emission filter >515 nm). All images were captured on Kodak Elite Chrome 400-slide film. The slides were subsequently scanned using a Nikon COOLSCAN Film scanner. The acep–gfp-containing M. smegmatis cells were imaged using a Photometrics PXL cooled CCD camera attached to a Nikon FX-A microscope with the standard Nikon FITC filter set. Some images were captured on an Olympus Fluoview 300 confocal microscope. All images were optimized using Adobe Photoshop 5.0.

Our efforts to permeabilize mycobacterial cells for immunolocalization of FtsZ using affinity purified M. tuberculosis anti-FtsZ antibodies were unsuccessful.

**Electron microscopy.** The cells were fixed in a mixture of 1:5% (v/v) glutaraldehyde and 1% (w/v) osmium tetroxide buffered with 0.1 M sodium cacodylate, pH 6.0 for 30 min at room temperature followed by 30 min at 0°C. The cells were then rinsed in deionized water and incubated with 0.5% (w/v) uranyl acetate at 0°C overnight, dehydrated in ethanol, embedded in Spur's plastic and sectioned. The sections were stained with Reynolds' lead citrate and viewed in a JEOL 1200EX electron microscope at an accelerating voltage of 60 kV. Images were captured on Kodak 4489 film.

**RESULTS**

**FtsZ levels at different stages of growth**

The intracellular concentration of FtsZ in E. coli appears to be an important factor in regulating its assembly into the Z ring. Basal levels of FtsZ in actively dividing cells of E. coli are very high (~20 000 molecules per cell) (Lu et al., 1998; Luktenhaus & Addinall, 1997; Margolin, 2000). It is unknown whether mycobacteria also contain high basal levels of FtsZ, as in E. coli, and whether their FtsZ levels alter with respect to growth stage. To address these questions, intracellular levels of FtsZ in wild-type M. tuberculosis and M. smegmatis cultures were determined by Western blot analysis. The M. tuberculosis FtsZ protein accounted for approximately 1.6, 1.0, 0.9 and 0.5% of the total protein during the exponential, late-exponential, stationary and late-stationary phases of growth, respectively (Fig. 1). These numbers correspond to approximately 30 000, 20 000, 9000 and 7000 molecules of FtsZ per cell, respectively. Similar growth-phase-dependent changes in FtsZ levels were noted in M. smegmatis, i.e. 1.0, 0.62, 0.42 and 0.4% of the total protein (Fig. 1) which correspond to 12 000, 7500, 2050 and 850 FtsZ molecules per cell, respectively. The relatively high levels of FtsZ during active growth and low levels during non-dividing or stationary growth tend to suggest a correlation between the intracellular concentration of FtsZ and active cell division. The differences in the absolute levels of FtsZ in the two mycobacterial species examined could be due to differences in the affinity of the M. tuberculosis FtsZ antibodies to the corresponding FtsZ proteins. Alternatively, it is possible that M. tuberculosis FtsZ antibodies have similar affinities to both M. tuberculosis and M. smegmatis FtsZ proteins and that the observed FtsZ levels in M. smegmatis are intrinsically low. As we do not have purified M. smegmatis FtsZ protein at this point we cannot distinguish between the two possibilities.

**Expression of M. tuberculosis ftsZ in M. tuberculosis**

To further understand the relationship between FtsZ levels and cell division, we attempted to express the M. tuberculosis ftsZ from heterologous mycobacterial pro-
motors and investigated the consequences of ftsZ overexpression on the levels of FtsZ protein, growth, viability and changes in cell morphology. The heterologous promoters used included two constitutively active promoters (the hsp60 and dnaA promoters) and the chemically inducible acetamidase promoter. Analysis of promoter activity by measuring the amount of GFP produced in a SLM-8000 fluorimeter (SLM-AMINCO) revealed that the activity of the hsp60 promoter in M. tuberculosis hosts was approximately fourfold higher than that of the dnaA promoter (data not shown). Furthermore, the M. avium dnaA promoter activity in M. tuberculosis hosts was approximately twofold higher than that in M. smegmatis hosts (data not shown). Thus, it is anticipated that expression of ftsZ from the dnaA and hsp60 promoters in merodiploid strains would result in elevated but differing amounts of FtsZ protein.

Several attempts to transform M. tuberculosis with hsp60p–ftsZ and dnaAp–ftsZ failed to produce any viable transformants. In one attempt with the hsp60p–ftsZ construct, four transformants were obtained at 30 °C, but plasmid analysis revealed deletions in the ftsZ coding region (data not shown). In contrast, the hsp60p–ftsZ construct containing a deliberately introduced 650 bp deletion in the ftsZ coding region transformed M. tuberculosis very efficiently (data not shown). From these data, we concluded that constitutive expression resulting in highly elevated levels of FtsZ in M. tuberculosis hosts is lethal. In the case of acep–ftsZ, although viable transformants were obtained, the resulting merodiploid strain produced inconsistent levels of FtsZ upon induction with acetamide (data not shown).

Consequently, the physiological effects associated with M. tuberculosis ftsZ expression from the ace promoter could not be evaluated. Presumably the conditions used to express ftsZ in M. tuberculosis hosts from the ace promoter were not optimal.

Expression of M. tuberculosis ftsZ in M. smegmatis

Considering the similarities in the composition of the cell division machineries of M. smegmatis and M. tuberculosis, and in view of the lethality associated with the M. tuberculosis ftsZ expression from the hsp60 and dnaA promoters, and the lack of consistent ftsZ expression from the ace promoter in M. tuberculosis hosts, we decided to evaluate the consequences of M. tuberculosis ftsZ expression in M. smegmatis. Both differences and similarities between M. tuberculosis and M. smegmatis hosts were noted. First, like the situation with the M. tuberculosis hosts, viable transformants were not obtained with the hsp60p–ftsZ construct. In one of the experiments with the hsp60p–ftsZ construct, a few transformants were obtained at 30 °C, but all of the recovered plasmids had deletions in the ftsZ gene. Plasmids bearing deliberately introduced internal deletions in the ftsZ gene resulted in normal transformation efficiencies (data not shown). Since the hsp60 promoter is active even at 30 °C, these results further support the notion that constitutive overproduction of M. tuberculosis FtsZ from the strong hsp60 promoter is toxic to M. smegmatis as well. Second, unlike the situation seen with M. tuberculosis, viable transformants were obtained with the dnaAp–ftsZ construct. Plasmid analyses confirmed the presence of the intact dnaA promoter and the ftsZ gene. Presumably, lower dnaA promoter activity in M. smegmatis enabled the recovery of viable transformants with the dnaAp–ftsZ construct.

The merodiploid strain expressing ftsZ from the dnaA promoter grew slowly both on agar plates and in liquid
culture, and exhibited an increased tendency to clump as compared to the controls (data not shown). Vortexing the cells in the presence of 1 mm glass beads followed by brief sonication disrupted these clumps. Protein analysis by Western blotting followed by quantitation on a Bio-Rad Molecular Imager revealed that the FtsZ protein levels in these cells were approximately sixfold higher than in controls (see Fig. 2a, compare lanes 1 and 2). Constitutive FtsZ overproduction resulted in ~1.0 log reduction in viability (data not shown) and delayed the appearance of colonies on agar plates by 2 days relative to the control (data not shown).

Expression from the inducible acetamidase promoter upon addition of acetamide resulted in approximately 22-fold FtsZ overproduction as compared to controls in 5 h (Fig. 2a, compare lane 7 with 6). Continued incubation beyond 12 h resulted in total cell lysis (data not shown), suggesting that elevated levels of M. tuberculosis FtsZ protein in M. smegmatis are lethal. Consistent with this finding, we found a reduction in viability of acep–ftsZ cells as compared to controls when plated on acetamide plates (Fig. 2b, see pSAR16). Analysis of plasmids recovered from cells grown on acetamide plates confirmed the presence of the intact ftsZ gene and ace promoter (data not shown). However, when tested for FtsZ overproduction, the respective cultures failed to produce FtsZ to any elevated levels (data not shown). To test whether the fisZ gene or the promoter accumulated mutations during growth on acetamide plates, the fisZ insert was replaced with a wild-type ftsZ gene and tested for protein overproduction by Western blotting. No changes in FtsZ protein levels were detected. Reciprocal experiments by cloning the recovered fisZ insert under the wild-type ace promoter resulted in elevated levels of FtsZ (data not shown). Together these results suggest that growth on plates containing acetamide leads to accumulation of mutations in the ace promoter region.

To investigate the morphological changes associated with sixfold FtsZ overproduction, the dnaAp–ftsZ merodiploid strains were visualized by light microscopy. Most of the cells (~83%) were long (filamentous), approximately 2–5 times the size of the control cells (Fig. 3, compare b and c with a), although some were normal in size. Some of the filamentous cells contained buds and branch-like outgrowths either in medial locations (arrowheads in Fig. 3b and 3c; see also Fig. 4, a and c) or in different positions along the length of the elongated cell (data not shown). Some cells contained bulbous structures at one end (arrowhead in Fig. 3b), but such structures at both ends were not seen. The buds could be initiating branch outgrowths. A few cells contained more than one outgrowth. Cell length measurements revealed that only 17% of the merodiploids were normal in size, i.e. 2–3 µm, and the remaining 83% of cells were long with varying sizes (Table 1). Less than 3% of the cells were >16 µm in length and ~12% had branches. The mean size of a branched cell was ~9 µm and the mean length of the outgrowths was about 1 µm. In contrast, ~10% were between 4 and 5 µm in size (Table 1, see vector control). Presumably, these cells were in the process of undergoing cell division. Under the light microscopy conditions used, we could not detect distinct septa or defined constriction-like structures. Hence, to further investigate the cell morphology and the nature of septa, thin sections of fixed cells of the

![Fig. 3. Effect of overproduction of M. tuberculosis FtsZ on cell morphology of M. smegmatis. Panels (a)–(f) are phase-contrast images captured using a 40x objective on a Zeiss photomicroscope. (a) pMV261 (control plasmid) cells, (b, c) pFR32 (dnaAp–ftsZ) cells, (d–f) pSAR16 (acep–ftsZ) cells grown in the absence (d) or presence of 1% acetamide (e, f). Arrows indicate bulbs/swellings (labelled 1) and branches (labelled 2).]
Mycobacterium tuberculosis FtsZ overproduction

Fig. 4. Transmission electron microscopy of M. smegmatis cells overexpressing ftsZ. Micrographs of ultrathin sections of M. smegmatis carrying the pFR32 vector (dnaAp–ftsZ) (a–e), or pSAR16 (acep–ftsZ) (f, g) or pJAM2 (control plasmid) (h) were viewed on a JEOL 1200EX electron microscope. The pSAR16 and pJAM2 cells were induced with 1% acetamide for 5 h for this experiment. Bars, 1 µm. Arrowheads indicate aberrant septa (labelled 1), branches (labelled 2) and buds (labelled 3).

Table 1. M. smegmatis cell length measurements upon M. tuberculosis FtsZ overproduction

The number of cells of a particular length (out of a total 100 cells measured in each case) are indicated. The vector alone is the control (pMV261 vector) for dnaAp–ftsZ cells.

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<th>Approximate size (µm)</th>
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<td>acep–ftsZ</td>
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<td></td>
<td>(– acetamide)</td>
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dnaAp–ftsZ merodiploids were examined by transmission electron microscopy (Fig. 4). Cells with abnormal septa, i.e. closely spaced and/or incomplete septa (Fig. 4a, b and d), and some with normal, medial septa (Fig. 4e) were readily apparent. Bud and branch-like structures were not separated from the main cell by septa (Fig. 4a and c). Extensive branching, as seen with the blockage of cell division due to inactivation of the whmD gene (Gomez & Bishai, 2000), was not observed. Together these results indicate that a sixfold overproduction of M. tuberculosis FtsZ affects septum formation and cell morphology in M. smegmatis.

The acep–ftsZ cells overproducing FtsZ protein were also filamentous as compared to uninduced controls (Fig. 3, compare e and f with d) and contained bulbous growths, bud and branch-like structures (see arrowheads in Fig. 3f). Nearly 94% of the induced cells were ~ 4 times the mean length of an uninduced cell whereas only 30% of the dnaAp–ftsZ cells were longer than control cells (see Table 1). Furthermore, 51% of the cells were 11–15 µm, 30% of the cells were 8–11 µm and 13% of the cells were greater than 16 µm long (Table 1). Approximately 21% of the elongated cells contained ~ 1 µm long branches and the mean size of the branched
Fig. 5. Localization of nucleoids inftsZ expressingM. smegmatis cells. The M. smegmatis cells expressingftsZ from the dnaA promoter (a) or the ace promoter (b), or those carrying the control vector pMV261 (c) were stained with a mixture of ethidium bromide and mithramycin as described in Methods. Cell morphology was visualized by DIC microscopy (upper panels) and the nucleoids were localized by fluorescence microscopy (lower panels).

Fig. 6. Localization of FtsZ inM. smegmatis overexpressingM. tuberculosisftsZ. The acep–ftsZ cells (pJFR11) were grown with shaking at 37 °C and induced with 1% acetamide for 5 h. In some cases cells were stained with a mixture of ethidium bromide and mithramycin as described in Methods. Cell morphology was visualized by DIC microscopy and FtsZ–GFP was localized by fluorescence microscopy. (a) DIC image of pJFR11 cells captured on an Olympus Vanox-T AH-2 microscope. (b) Fluorescence image of the same view as (a). (c) Fluorescence image of an FtsZ–GFP producing cell captured on an Olympus Fluoview 300 confocal microscope. (d) The same view as (c) with stained nucleoids. (e) M. smegmatis cells with the control vector, pJFR14, imaged using a Photometrics PXL cooled CCD camera attached to a Nikon FX-A microscope and the standard Nikon FITC filter set.

Visualization of FtsZ

To evaluate the fate of M. tuberculosis FtsZ in the filamentous cells, the M. tuberculosis FtsZ protein was expressed as a fusion protein tagged with GFP and was visualized by fluorescence microscopy. These experiments were carried out only with acep–ftsZ–gfp construct (pJFR11), as fluorescence with the dnaAp–ftsZ–gfp construct was not high enough to visualize any FtsZ–GFP structures. Independent experiments revealed that the pJFR11 cells (acep–ftsZ–gfp) behaved the same as pSAR16 (acep–ftsZ) with respect to FtsZ overproduction (data not shown), production of filamentous cells (Fig. 6a) and inhibition of growth upon induction.
with acetamide (data not shown). Examination by epifluorescence illumination revealed multiple uniformly spaced green fluorescent regions in acetamide-induced acep–ftsZ–gfp cells (Fig. 6b). It is not clear at this point how much of the observed growth-inhibitory effects in these cells are due to the presence of the GFP tag. Further characterization of ftsZ–gfp in different genetic backgrounds is required to address this question. Cells that overproduced GFP alone from the ace promoter were normal in size and were uniformly fluorescent (Fig. 6c), suggesting that the GFP is distributed throughout the bacterial cell. No FtsZ–GFP bands were detected under non-inducing conditions, i.e. in the absence of acetamide (data not shown), despite the fact that a small amount of FtsZ–GFP fusion protein was detected by Western analysis (data not shown). Presumably, the amount of FtsZ produced was insufficient to detect any visible FtsZ structures by fluorescence microscopy. Together these data indicate that the FtsZ protein is responsible for the formation of the organized structures. Since the FtsZ–GFP structures were not randomly distributed in the cell, but rather located at a uniform distance from each other, we believe that these structures delineate putative FtsZ-nucleation sites. In an effort to distinguish whether the observed FtsZ–GFP structures are aggregates or specific Z bands/ring-like structures, induced acep–ftsZ–gfp cells were examined by confocal microscopy. Although ring-like structures could not be resolved, we were able to visualize distinct FtsZ foci flanking the nucleoids (Fig. 6d). These data also indicate that filamentous cells are multinucleoidal.

**DISCUSSION**

We have shown that the FtsZ protein levels in both *M. tuberculosis* and *M. smegmatis* are growth-phase dependent (Fig. 1) and that elevated intracellular levels of *M. tuberculosis* FtsZ protein interfered with the cell division process and inhibited growth. Transmission electron microscopy of FtsZ-overproducing cells showed both abnormal and multiple septa, and the presence of buds and branches. Finally, FtsZ-localization experiments using acep–ftsZ–gfp revealed distinct FtsZ foci interspersed with nucleoids. Together, these results suggest that elevated levels of *M. tuberculosis* FtsZ protein interfere with steps subsequent to the formation of FtsZ-organized structures (Fig. 5) and that the intracellular concentration of FtsZ is one of the important parameters regulating the cell division process in mycobacteria.

The observed growth-phase-dependent changes in the intracellular levels of FtsZ lead us to conclude that a certain threshold concentration of FtsZ is required for active cell division. Although the intracellular levels of FtsZ decreased during the nonreplicating growth period, they still accounted for ~0.5% of the total cellular protein. Similar results were also noted with *M. smegmatis* cells. These high levels suggest that either *M. tuberculosis* FtsZ is continuously synthesized at a reduced rate or it exhibits a slow turnover rate in the nonreplicating cells. Alternatively, nonreplicating cells may contain some unresolved FtsZ-associated structures. Recently it has been shown that dormant cultures of *M. tuberculosis* exhibit both transcriptional and metabolic activity (Hu et al., 2000). It is unknown whether such transcriptional activity extends to the ftsZ gene. Further experiments are required to address these questions.

The genetic and biochemical aspects of the initiation of cell division in mycobacteria are largely unknown and much of our knowledge of the role of FtsZ in the initiation of cell division comes from studies carried out with *E. coli*. In the case of *E. coli*, up to a sevenfold overproduction of FtsZ does not increase cell length but produces minicells. Furthermore, minicell production does not occur at the expense of normal cell division (Ward & Lutkenhaus, 1985). FtsZ overproduction beyond sevenfold leads to filamentation and subsequent inhibition of growth. We noted distinct phenotypic effects associated with *M. tuberculosis* FtsZ overproduction. First, unregulated expression of ftsZ from either the strong hsp60 promoter or a fourfold less active *M. avium* dnaA promoter was lethal to *M. tuberculosis*, whereas only expression from the strong hsp60 promoter was lethal to *M. smegmatis*. Presumably, the cell division process in the slow growers is more sensitive to perturbations in FtsZ levels than in the rapid growers. The *M. smegmatis* dnaAp–ftsZ merodiploids exhibited an increased tendency to clump in broth and produced buds and branches. Absence of extensive branching suggests that these cell wall outgrowths are transient structures and the respective cells either lyse or recover from branching following cell division. Our present data do not distinguish between the two possibilities.

The genetic elements responsible for clumping in *M. smegmatis* are unknown. It has been shown recently that mutations in *mpA*, one of the genes involved in the phospholipid biosynthesis pathway of *M. smegmatis*, also causes clumping (Parish et al., 1997). Assuming that the formation of Z ring (FtsZ structures) in mycobacteria marks the beginning of the septation process, then a sixfold overproduction of FtsZ which resulted in the formation of aberrant septa (Fig. 4a, b and d) could, in turn, affect the downstream events and the co-ordination between Z ring assembly and cell wall synthesis. In this situation changes in phospholipid biosynthesis might result which could in turn induce clumping.

Regulated expression of *M. tuberculosis* ftsZ from the ace promoter in *M. smegmatis* was also toxic and resulted in production of filamentous cells. Many of these filamentous cells lacked any visible septa (Fig. 5) but contained defined FtsZ foci at discrete locations interspersed with distinct nucleoids. These results suggest that in *M. smegmatis* and possibly in *M. tuberculosis*, DNA replication and segregation can continue to proceed even in the absence of cell division and that the observed filamentation phenotype is due to blockage of cell division at steps subsequent to the formation of organized FtsZ structures. Overproduction...
of FtsZ, without concomitant overproduction of other cell division components could lead to an imbalance in the cell division machinery which might in turn inhibit cell division. Indeed, simultaneous overexpression of other cell division genes relieves the block in cell division that accompanies the overexpression of ftsZ in E. coli (Dai & Lutkenhaus, 1992). It remains to be tested whether the same is true for mycobacteria.

In Rhizobium meliloti, overproduction of either homologous or heterologous FtsZ produces branched structures (Latch & Margolin, 1997). These cell wall outgrowths are often confined to the medial locations, suggesting that cell walls are breached at the mid cell locations. Branching is also observed in E. coli cells constitutively expressing ftsZ84 in a min mutant background (Yu & Margolin, 2000). Unlike the situation with R. meliloti, we found that branches and buds were present at random locations and were not separated by septa from the main cell mass. Presumably, in the absence of productive septum formation these outgrowths are produced to accommodate cell mass increase in the merodiploid cells.

The ace promoter used to drive expression of ftsZ is derived from the M. smegmatis amide operon (Triccas et al., 1998) and the M. tuberculosis genome lacks this region (Cole et al., 1998). While this promoter system has been used to express some genes in M. tuberculosis, e.g. sigA, our experience indicated that not all genes cloned downstream of the ace promoter are well expressed in M. tuberculosis hosts, e.g. ftsZ (this study), dnaA and ercc-3 (M. V. S. Madiraju, R. Wilson, J. Dziadek & M. Rajagopalan, unpublished data). Optimal expression from the acetamidase promoter is believed to require its upstream regulatory sequences (T. Parish, personal communication). Thus, the observed large scale overproduction of M. tuberculosis FtsZ in M. smegmatis hosts is due to in trans activity of these regulatory sequences present on the M. smegmatis genome, which is a limiting factor in the case of expression in M. tuberculosis hosts. Thus, either optimization of amide promoter activity in M. tuberculosis hosts or the use of different inducible promoter systems such as the isocitrate lyase promoter system which is being constructed (D. G. Russell, personal communication), could allow us to investigate intermediary levels of expression of ftsZ between that from the ace promoter and the presumably lethally high levels from the dnaA and hsp60 promoters.

The ability of the M. smegmatis dnaAp–ftsZ strain to survive and multiply, albeit aberrantly, suggests that the M. tuberculosis ftsZ is functional and participates in the cell division process of M. smegmatis. This is not an unreasonable assumption considering the sequence similarity of the two FtsZ proteins and the similar genetic composition of the putative cell division machineries of the two organisms. Whether or not the two species contain other protein(s) that are specific to their individual cell division processes needs to be determined by further studies. An alternative possibility that the normal septa in these merodiploids result from the FtsZ ring formed from the M. smegmatis protein cannot be ruled out at present. Sequence analyses grouped FtsZ protein into three domains: one highly conserved N-terminal domain of ~ 316 aa; a core linker region of variable sequence and length, and a highly conserved short C-terminal tail. The N-terminal region of FtsZ is believed to be important for GTP-dependent polymerization activity whereas the C-terminal region is critical for interaction of FtsZ with other cell division proteins (Ma et al., 1996; Ma & Margolin, 1999; Margolin, 2000). The continuous but slow growth of dnaAp–ftsZ cells tends to suggest that the observed differences in the C-terminal region between the two FtsZ proteins, while perhaps important, are not sufficient to block the cell division process. Nonetheless, these results indicate that the M. tuberculosis FtsZ protein plays a critical role in its cell division and that the in vivo FtsZ levels influence or affect the number and nature of septa formed.

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