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Mycobacterium tuberculosis ftsZ expression and minimal promoter activity

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SUMMARY

Optimal levels of *ftsZ* gene product are shown to be required for initiation of the cell division process in *Mycobacterium tuberculosis*. Here, we report that the *fts*Z gene expression is sharply down-regulated during starvation and hypoxia, conditions that are believed to result in growth arrest, but is restored upon dilution of cultures into fresh oxygen-rich media. Primer extension analysis identified four transcriptional start sites, designated as P1, P2, P3 and P4 at nucleotide positions −43, −101, −263, and −787, respectively, in the immediate upstream flanking region of the *fts*Z initiation codon. Promoter deletion and homologous recombination experiments revealed that *fts*Z expression from the 101-bp region is sufficient for *M. tuberculosis* viability. All promoter strains had reduced FtsZ levels compared to wild-type, although the loss of P4 severely compromised FtsZ levels during both the active and stationary phases. We propose that *fts*Z expression from all promoters is required for optimal intracellular FtsZ levels and that the activities of P4 and possibly other promoters are down-regulated during growth arrest conditions.

Keywords

FtsZ; Mycobacterium; cell division; transcription

1. Introduction

A hallmark of tuberculosis is latency, wherein *Mycobacterium tuberculosis*, the causative agent, is believed to remain in a non-proliferative persistent (NRP) state with limited bacterial turnover during its growth in granulomas[1, 2]. It is believed that *Mtb* encounters hypoxic conditions in granulomas[3, 4]. *Mtb* cultures exposed to oxygen-deprived conditions in nutrient broth attain NRP state, but upon resuspension in oxygen-rich media undergo a round of cell division prior to initiating new rounds of DNA replication[2, 5]. These results suggest that the cell division process is tightly regulated during hypoxia or NRP state.

FtsZ is an essential protein critical for the initiation of cell division in nearly all prokaryotes and in some mitochondria and chloroplasts[6, 7]. FtsZ protein polymerizes in a GTP dependent manner and forms a contractile cytokinetic ring or the Z-ring at a pre-determined septal site, usually at the mid-cell position[6, 8, 9]. Apart from recruiting as many as 13 additional cell division proteins to the division site, FtsZ is likely to provide the force for constriction of the septum in the dividing cell[7, 10–13]. FtsZ of *M. tuberculosis* ($FtsZ_{TB}$), although similar in structure and sequence to other prokaryotic FtsZ proteins, exhibits slow polymerization and weak GTPase activities[14, 15]. *M. tuberculosis* FtsZ protein levels decrease during stationary

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growth and optimal $FtsZ_{TB}$ levels are needed to sustain cell division[16–18]. Investigations on the kinetics of FtsZ protein assembly dynamics revealed that the half-time for subunit turnover is 42 sec in vitro and 25 sec in vivo[19]. Together, these findings suggest that *fts*Z expression is subject to regulation in *M. tuberculosis* and that characterization of *fts*Z promoter region could provide clues in this direction.

The immediate upstream flanking genes of *M. tuberculosis fts*Z include *fts*Q and *mur*A and the intergenic region between *fts*Z and *fts*Q is 174-bp. One earlier report identified several transcriptional start sites in the promoter region of *fts*Z[20]. It is unknown if *fts*Z expression from all these promoters is required for cell division and if there is a minimal promoter region needed to sustain cell division and growth. Furthermore, it is unknown if there is any correlation between the FtsZ levels and the expression from individual promoters. The present study addresses these questions.

2. Materials and methods

2.1. Bacterial growth conditions

Escherichia coli strains were grown in Luria–Bertani (LB) broth or agar supplemented with kanamycin (Km-50 μg ml^{-1}) or hygromycin (hyg-50μg ml^{-1})[17]. *M. tuberculosis* strains were propagated in Middlebrook 7H9 broth supplemented with OADC (oleic acid, albumin, dextrose, catalase with sodium chloride) and plated on 7H10 plates containing appropriate antibiotics (Km at 25 μg ml^{-1}; Hyg at 50 μg ml^{-1}). Growth was monitored by absorbance at 600 nm and viability by determining colony forming units on Middlebrook 7H11 agar plates (Remel).

2.2. Construction of recombinant ftsZ promoter strains

The Hyg^r pJfr66 plasmid carrying *ftsZ* and its upstream 1 kb region contains four *fts*Z promoters referred to as P1-P4 and was used as a template to generate DNA fragments containing the *fts*Z coding region with different lengths of promoters[21]. The P1 fragment carries promoter 1, P2 carries promoters 1-2, P3 carries promoters 1-3 and P4 carries promoters 1-4. The promoter fragments were cloned individually into pMV306K, a Km^r integrating vector (Table 1), and confirmed by sequencing. The plasmids with the respective promoter fragments were named as pMK1, pMK2 and pMK3 (Table 1) and were used to exchange the resident pJFR66 in *Mtb-66* by transformation as described[21]. Genomic DNA from all recombinant strains was extracted; the *fts*Z region was amplified and verified by sequencing.

2.3. Western blotting

FtsZ levels were quantitated by immunoblotting and normalized to MtrA as the levels of the latter are shown to be constant under various growth conditions[22].

2.4. RNA extraction, primer extension and quantitative real-time (QRT) PCR

Extraction of total RNA and determination of *fts*Z transcript levels relative to house keeping 16S rRNA gene by quantitative real-time (qRTR-PCR) were essentially as described[22, 23]. The reactions were performed in triplicate on at least two independent RNA preparations. Primer extension was carried out using 10 to 20 μg total RNA, γ-^{32}P end-labeled oligonucleotide primers specific to *fts*Z upstream region and AMV reverse transcriptase utilizing the primer extension system from Promega. For each primer extension reaction a DNA sequencing ladder was generated with pJFR66 DNA as the template and the corresponding primer used for primer extension (not shown). DNA sequencing was done using the Sequenase Version 2.0 DNA sequencing kit from USB, the products were resolved

on 10% denaturing acrylamide gels, and processed for autoradiography. Bands were visualized using the BIORAD Molecular Imager and analyzed by QuantityOne software.

2.5. Construction of ftsZ promoter–luciferase (lux) plasmids and measurement of lux activity

Different lengths of the 5' flanking region of $ftsZ_{tb}$ bearing appropriate promoters/ transcriptional start sites were PCR amplified, cloned upstream of the promoter-less *lux* gene in pMV306K vector and transformed into *M. tuberculosis*. Luciferase activity was measured by mixing 100 μL of exponentially growing cultures with 100 μL of Beetle Luciferin (1mM; Promega) and measured in a luminometer (Turner Designs). Luciferase activity is expressed as lux units per $O.D._{600}$.

2.6. Microscopic analysis and cell length measurement

M. tuberculosis cells were visualized on a Nikon Eclipse 600 microscope equipped with a 100X (Nikon Plan Fluor) oil immersion objective with a numerical aperture of 1.4 and cell lengths were determined as described[21].

3. Results and discussion

3.1. ftsZ expression is reduced during starvation and hypoxia

In an effort to understand if the *fts*Z expression is altered under conditions that lead to growth arrest, we measured the *fts*Z transcript levels relative to exponential growth phase by QRT PCR. The *fts*Z transcript levels were reduced 10-fold during stationary phase and hypoxia relative to the exponential phase, but were restored to wild-type levels following resuspension of the hypoxic cultures in fresh broth (Fig. 1). Since hypoxic cultures are blocked at the cell division step, these results suggest that *fts*Z gene expression is growth-phase dependent and that optimal *fts*Z transcript levels are necessary for the initiation of cell division.

3.2. Multiple ftsZ promoters contribute to ftsZ expression

To begin understanding how *fts*Z transcription is regulated, we extracted total RNA and carried out primer extension using oligonucleotide primers specific to the 1 kb *fts*Z upstream region. Four transcriptional start sites designated as P1, P2, P3 and P4 at −43, −101, −263 and −787 nucleotides, respectively, relative to the *fts*Z start codon were identified (Table 1; Fig. 2A). The last 2 promoters, P3 and P4, were located in the *fts*Q-coding region whereas those of the P1, and P2 were in the *fts*Q-*fts*Z intergenic region. While these results are in general agreement with those reported by Roy et al. ([20]), differences with respect to specific location of transcriptional start sites are noted (see Fig. 2A legend). These data, however, do not rule out the possibility of other regulatory elements or unidentified distant promoters present upstream of the 1 kb region that could affect *ftsZ* transcription.

*fts*Z promoter-luciferase fusion reporter gene expression analysis revealed that a construct bearing all four promoters showed the highest promoter activity whereas that containing P1 alone showed the least (Fig. 2B). DNA fragment containing the P2 and P1 promoters contributed to nearly 62% of total activity. The relative contribution of the four promoters was calculated to be: P1 - 5%, P2 - 57%, P3 - 14% and P4 - 23% with P2 being the most active promoter.

3.3. ftsZ expression from P2 promoter is sufficient for viability

M. tuberculosis FtsZ is an abundant protein with ~ 14, 000 molecules per cell[17]. To understand if *fts*Z expression from all four promoters is needed for cell division and optimal

FtsZ levels, we created Km^r plasmids expressing *fts*Z from DNA fragments bearing P1 (pMK1), P1+P2 (pMK2), P1+P2+P3 (pMK3) and used them to exchange the integrated Hyg^r plasmid pJFR66 expressing *fts*Z from P1-P4 promoters in *M. tuberculosis ΔftsZ* strain, *Mtb-66*[16]. Transformation of *Mtb-66* with pMK2 and pMK3, but not that with pMK1, produced viable transformants indicating that *fts*Z expression from a region containing P1 and P2 is sufficient to support growth and viability (not shown). DNA sequencing following PCR amplification confirmed that pMK2 and pMK3 had indeed replaced the resident plasmid pJFR66 in these experiments (not shown). One representative transformant each, designated as Mtb-pMK2 and Mtb-pMK3, respectively, was further characterized.

The recombinant strains Mtb-pMK2 and Mtb-pMK3, like the Mtb-66, showed reduced growth rate and viability as compared to the parent strain (Fig. 3, see inset). However, the growth rates of the Mtb-pMK2 and Mtb-MK3 were comparable to that of Mtb-66 (Fig. 3). Differences in the growth rate and viability between the wild-type and the recombinant promoter strains indicate that the sequences upstream of the 1-kb promoter region are necessary for optimal growth and viability. Alternatively, the location of *ftsZ* at the *attB* site may have affected the transcription of genes present downstream of *ftsZ*. Nonetheless, these data indicate that transcription from a fragment containing promoters 1 and 2 is sufficient for the viability of *M. tuberculosis*. Consistent with the slow growth rate, a modest 15% increase in average cell length of the recombinant strains was noted (data not shown).

3.4. FtsZ levels and the contribution of promoters

Next, we examined the FtsZ levels in the promoter strains during exponential and the stationary growth phases (Fig. 4). The following observations were evident: (1) FtsZ levels were reduced in the stationary phase relative to the active phase in the wild type strain. These results are consistent with the *mRNA* expression (Fig. 1) and our earlier published data[17]. (2). Expression of *fts*Z from *att*P site in the *Mtb-66* strain led to a minor reduction in the FtsZ levels under both growth conditions (Fig. 4. compare P1-4 with WT, also see inset). (3) Removal of the P4 promoter led a significant reduction in the FtsZ levels (Fig. 4 and inset, compare P1-3 with WT, P1-4 and P1-2). Simultaneous removal of P4 and P3 promoters restored FtsZ levels, albeit partially (see P1-2 in Fig. 4 and inset).

The presence of multiple functional promoters in the upstream flanking region of the *M. tuberculosis ftsZ*, as in other bacteria, signals a complex regulation[24–28]. These results suggest that while hitherto unidentified regulatory elements present upstream of the 1-kb promoter region are important for optimal *ftsZ* expression, the absence of P4 promoter activity significantly reduces the intracellular FtsZ levels. It is possible that under the conditions that promote growth arrest, *fts*Z expression from P4 and or more promoters is selectively prevented thereby resulting in decreased transcription and translation. Thus, decrease in *ftsZ* transcript levels during hypoxia and starvation and upregulation upon reactivation signals that *ftsZ* transcriptional regulatory mechanisms are at work under growth arrest conditions. We can also envision that intracellular FtsZ levels could be regulated at the protein level. For example, a recent report indicates that FtsZ is a substrate of FtsH protease in vitro[29]. Our preliminary data indicate that FtsZ levels are modulated in *M. tuberculosis* strains expressing altered levels of FtsH [30]. Presumably, FtsH and other unrecognized proteases, could act to reduce FtsZ levels. Recent experiments to determine FtsZ dynamics by fluorescent recovery after photo-bleaching (FRAP) technique revealed that only ~30% of the available intracellular FtsZ is engaged in the formation of septal Z-rings at midcell sites indicating that cell division can proceed with reduced levels of FtsZ[31, 32]. We envision that a combination of transcriptional and translational regulation controls FtsZ levels and therefore Z-ring assembly and cell division during normal growth, NRP state[2] and possibly during growth in granulomas.

Acknowledgments

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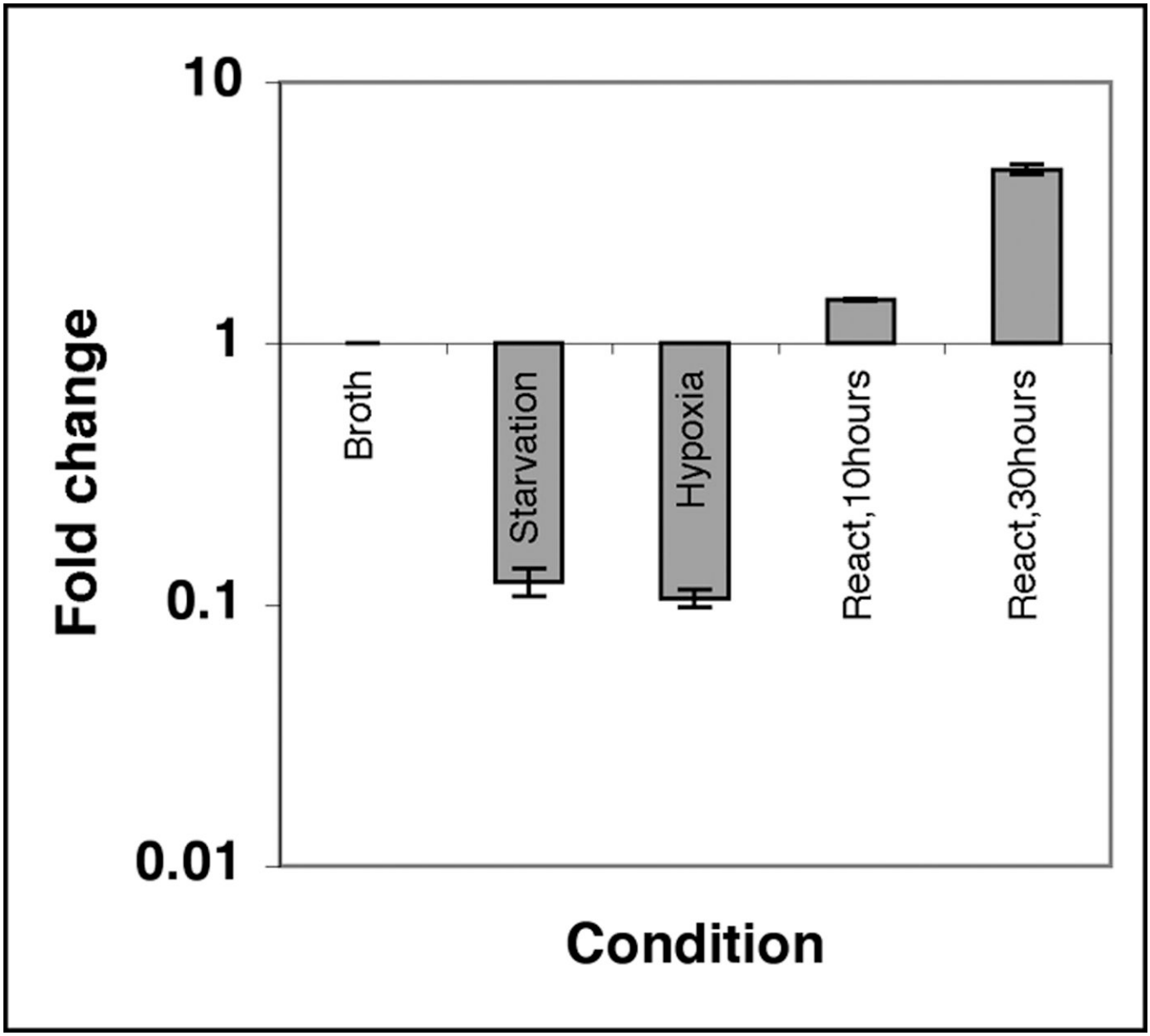

Figure 1.
Expression levels of *ftsZ* under different growth conditions. *M. tuberculosis* was grown in broth under various indicated conditions as described. RNA was extracted and levels of *ftsZ* mRNA were measured by quantitative real-time PCR using Taqman chemistry (see Table 1). The expression levels of *ftsZ* mRNA were normalized to 16s mRNA levels. Mean ±SD from three independent experiments are shown.

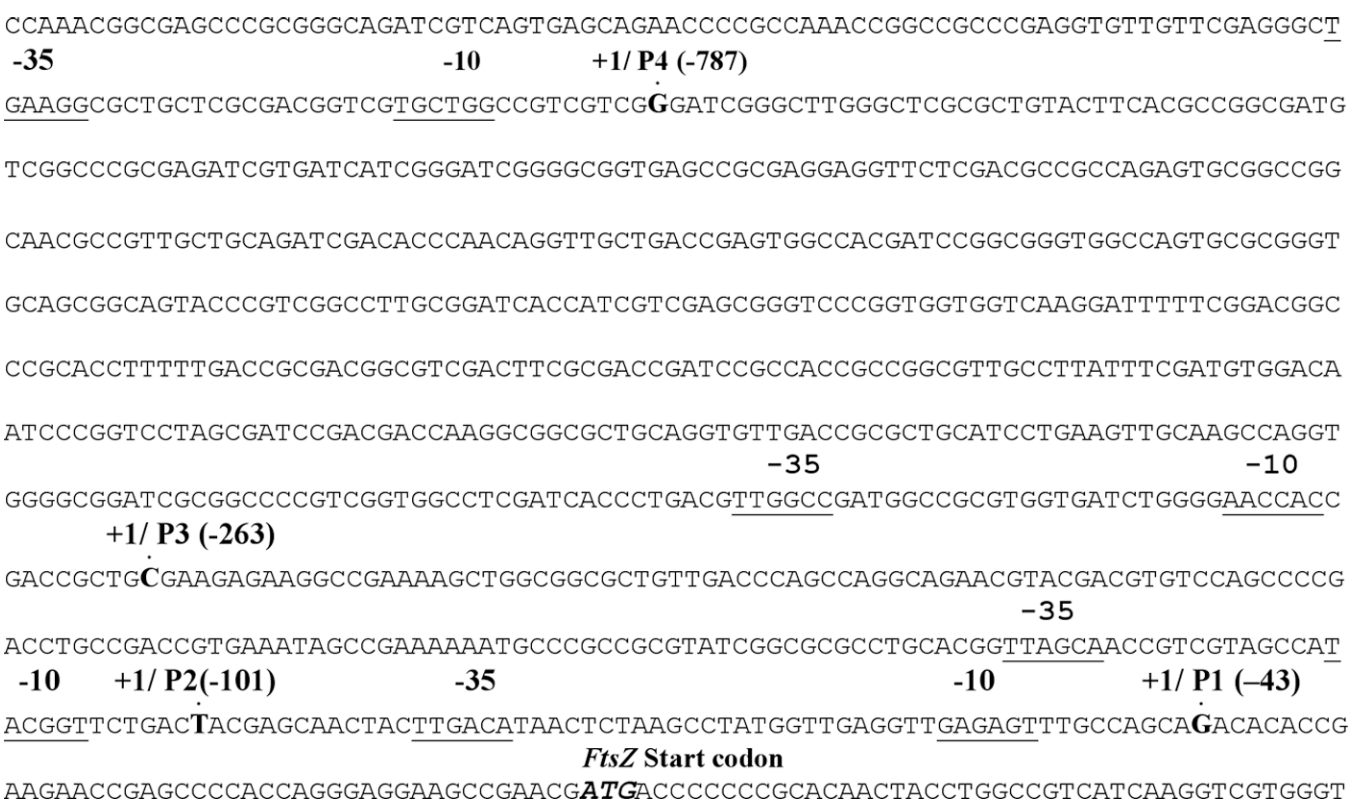

Figure 2.

Putative transcription start sites for *ftsZ*$_{TB}$. Transcription start sites and the putative −35 and −10 regions in the 5' flanking region of *ftsZ*$_{TB}$ are shown. *E. coli* sigma 70 type −35 and −10 boxes, TTagCA and TAcggT, respectively (uppercase - identity with the consensus), identified upstream of the P2 promoter. Because of at least 3 matches to the s^{70} *E. coli* consensus, the P2 promoter was included in group A mycobacterial promoters. For promoters P1 and P4, distinct s^{70} type −35 regions were seen, but no identifiable −10 sequences were found. For promoter P3, no apparent −10 or −35 regions were seen. Therefore, these three promoters could belong to mycobacterial group C promoters and may contain recognition sequences for regulators other than σ^A or σ^B ([33]).

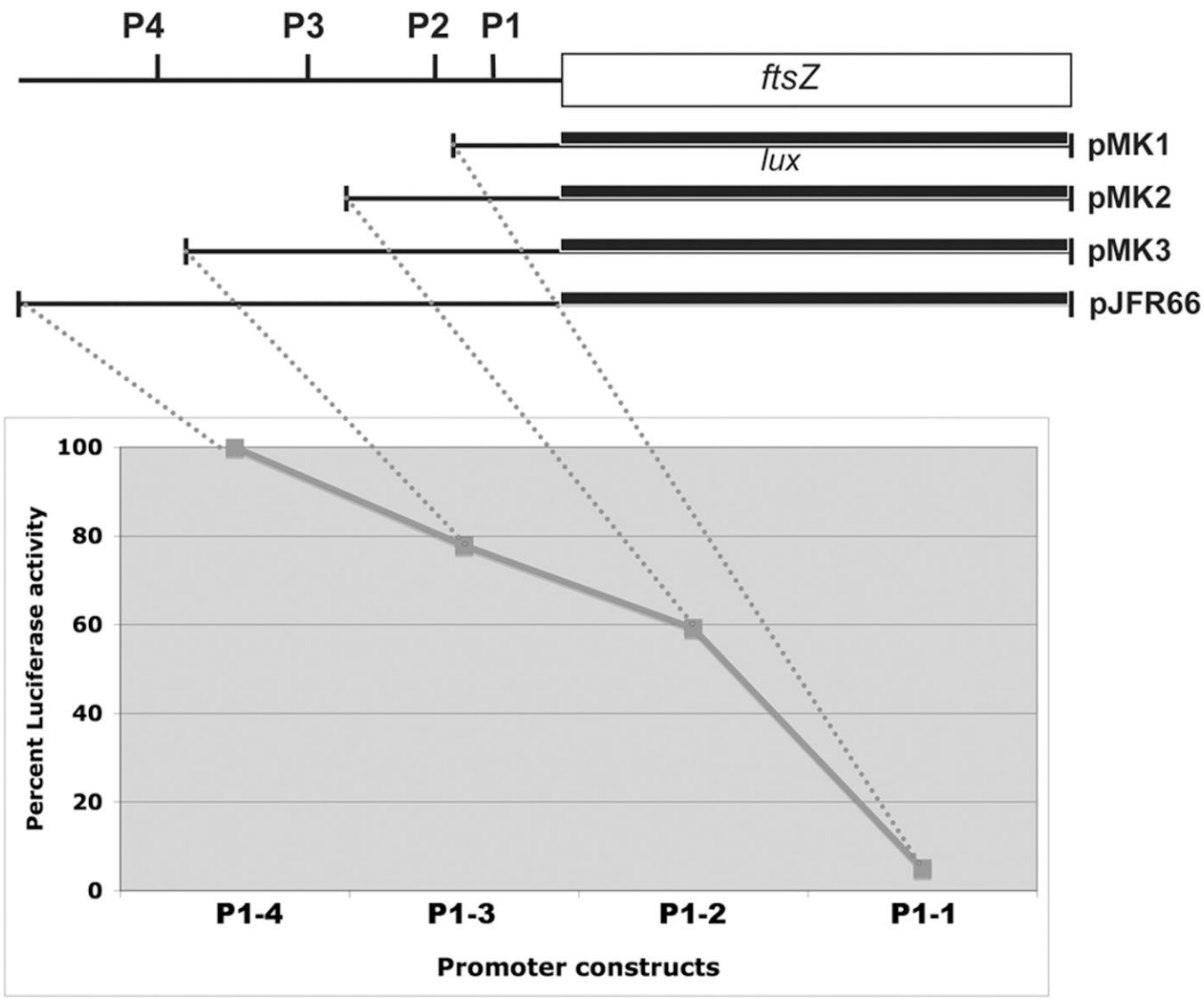

Figure 3.

Transcription from *ftsZ*$_{TB}$ promoters P1-P4. Transcriptional fusions bearing P1 to P4 promoters upstream of the *lux* gene in pEM45 (pRM15 – pRM18) were transformed to *M. tuberculosis* (upper panel) and luciferase assays were carried out using exponentially growing cultures of the recombinant strains. Lux units were determined and normalized to OD_{600} units (lower panel).

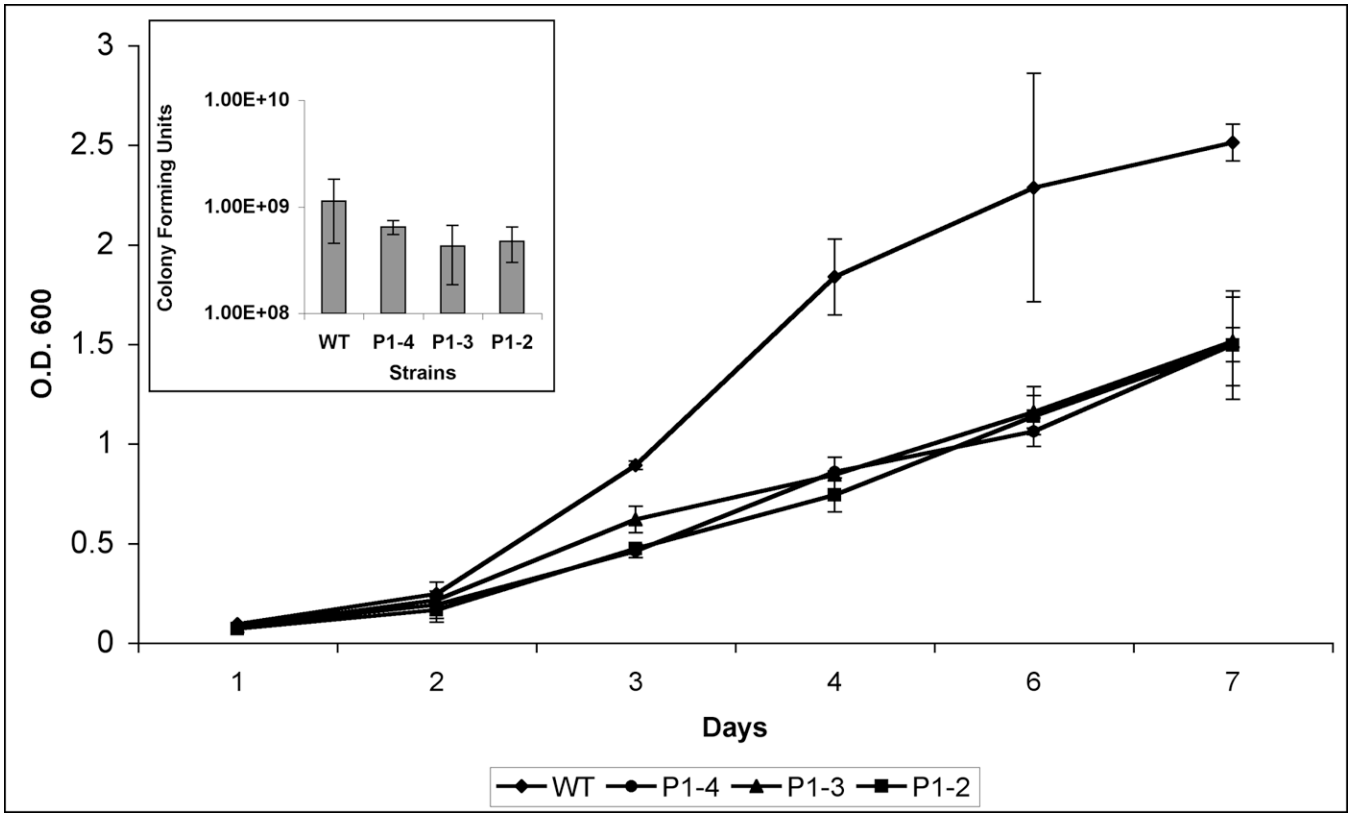

Figure 4.

Growth and viability of *M. tuberculosis ftsZ* promoter strains. *M. tuberculosis* H37Ra strains, Mtb-66 (P1-4), Mtb-pMK3 (P1-3) and Mtb-pMK2 (P1-2) were grown in 7H9 broth for indicated time-points and OD600 was measured and plotted. Mean ±SD from three independent experiments are shown. Inset: Exponential cultures from each strain were plated on 7H10 agar and CFU determined after 3 weeks. WT – *M. tuberculosis* H37Ra strain.

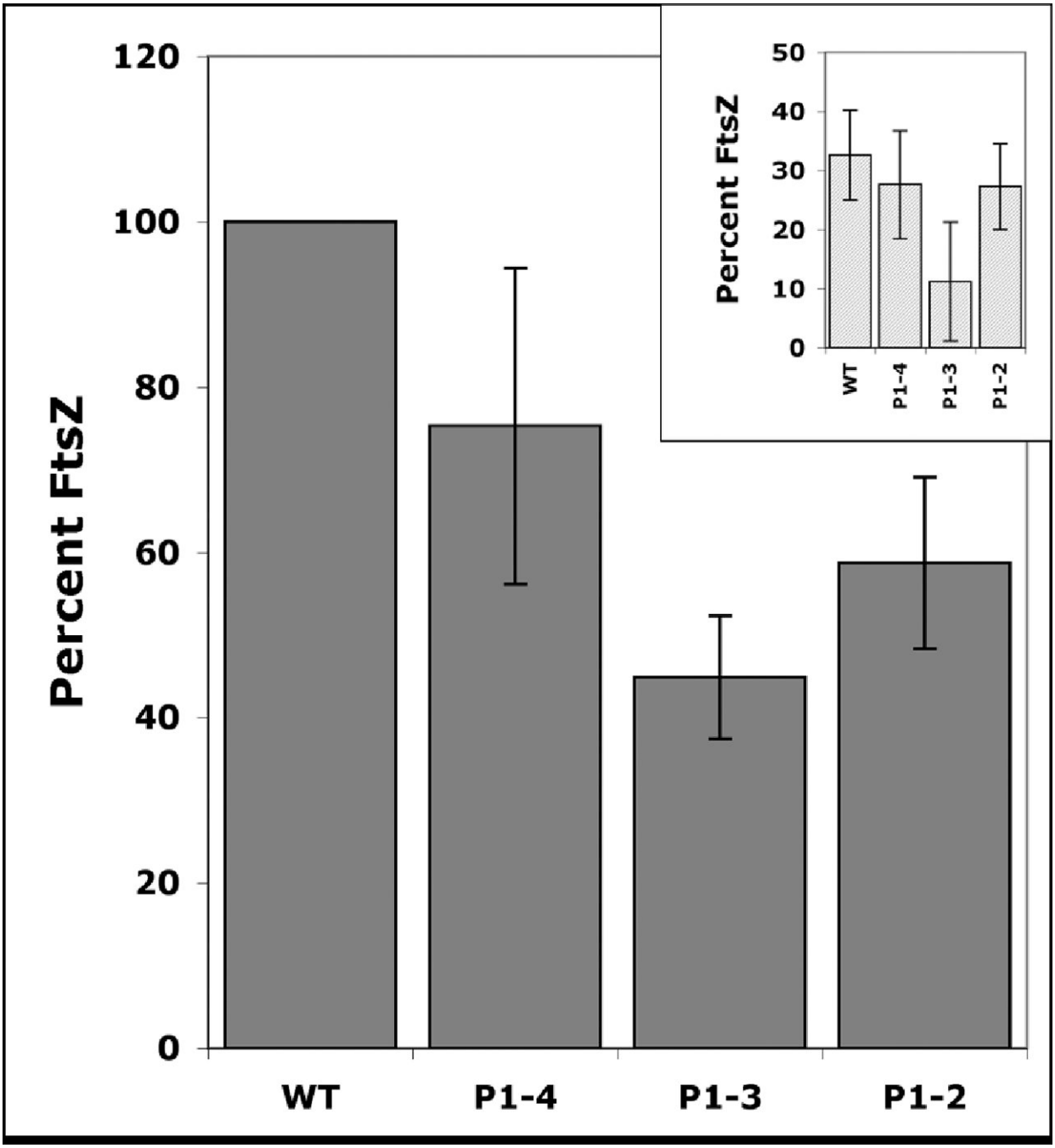

Figure 5.

FtsZ levels in *M. tuberculosis ftsZ* promoter strains: Exponential or stationary phase cultures of Mtb-66 (P1-4), Mtb-pMK3 (P1-3) and Mtb-pMK2 (P1-2) were lysed and separated on SDS-PAGE as described in Methods. Proteins were transferred to nitrocellulose membranes and Western blot analysis was carried out as described in Methods. FtsZ levels were measured and normalized to MtrA[22]. FtsZ levels in various promoter strains are expressed with respect to exponential phase wild-type *M. tuberculosis*. Inset: FtsZ levels in stationary phase.

Table 1

Plasmids	Description	Reference
pMH66	Mycobacterial integrating vector with luciferase gene, hyg^r	Park et al.
pEM43	pMH66 with 1 kb 5'flanking region of $ftsZ_{tb}$, hyg^r	This study
pEM45	3 kb *BamH*I-*Cla*I fragment from pEM43 in pMV206, km^r	This study
pRM15	278-bp flanking 5'$ftsZ_{TB}$ in pEM45, km^r	This study
pRM16	114-bp flanking 5' $ftsZ_{TB}$ in pEM45, km^r	This study
pRM17	804-bp flanking 5' $ftsZ_{TB}$ in pEM45, km^r	This study
pRM18	1068-bp flanking 5' $ftsZ_{TB}$ in pEM45, km^r	This study

Table 2

Oligo name	Oligo sequence	Description
MVM535	5’- CCT TGA TGA CGG CCA GGT AG - 3’	For mapping P1 by PE
MVM238	5’- GCG GAT CCG CTT CCT CCC TGG TGG GGC- 3’	For mapping P2 by PE
MVM534	5’- CGG GGC TGG ACA CGT CGT ACG - 3’	For mapping P3 by PE
MVM533	5’- CCT CCT CGC GGC TCA CCG CC - 3’	For mapping P4 by PE
MVM542	5’- CGC GGA TCC GAC TAC GAG CAA CTA CTT GAC -3’	For cloning P1
MVM541	5’- CGC GGA TCC CCG CTG CGA AGA GAA GGC CG -3’	For cloning P2
MVM540	5’- CGC GGA TCC CCG TCG TCG GGA TCG GGC TTG -3’	For cloning P3
MVM461	5’- TGG CAA CAC TAG TAC GAG GAGGC GGT TAC GGA G -3’	For cloning P4