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Brief note

Identification and functional annotation of mycobacterial septum formation genes using cell division mutants of *Escherichia coli*

Sujata S. Gaiwala Sharma[1], Vimal Kishore[1], Tirumalai R. Raghunand*

CSIR-Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, India

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Abstract

The major virulence trait of *Mycobacterium tuberculosis* is its ability to enter a latent state in the face of robust host immunity. Clues to the molecular basis of latency can emerge from understanding the mechanism of cell division, beginning with identification of proteins involved in this process. Using complementation of *Escherichia coli* mutants, we functionally annotated *M. tuberculosis* and *Mycobacterium smegmatis* homologs of divisome proteins FtsW and AmiC. Our results demonstrate that *E. coli* can be used as a surrogate model to discover mycobacterial cell division genes, and should prove invaluable in delineating the mechanisms of this fundamental process in mycobacteria.

Keywords: *Mycobacterium tuberculosis*; *Mycobacterium smegmatis*; *Escherichia coli*; Divisome assembly; Complementation

1. Introduction

One of the key factors contributing to the success of *Mycobacterium tuberculosis* as a pathogen is its ability to persist within an immunocompetent human host in a dormant form [4] and reactivate under favourable conditions such as weakened immunity or high oxygen levels [30]. Since cell division is intimately linked to metabolic shutdown, elucidating the mechanism of mycobacterial cell division is likely to provide critical insights into cellular processes guiding the shift-down of bacilli from a state of active division to dormancy.

The mycobacterial cell division process shows a few mechanistic variations compared to those described in *Escherichia coli* [11]. Cell division is initiated by FtsZ polymerization guided by interactions with structural components of the divisome, FtsW, FipA, CrgA and CwsA [17]. The predicted *M. tuberculosis* FtsW has been shown to interact with FtsZ and PBPB, a PG synthase, thereby forming a link between the Z-ring and peptidoglycan (PG) synthesis machinery [6]. This machinery in mycobacteria is composed of PbpB, PbpA and PonA1 [5,13]; thus far RipA, an endopeptidase, and its interacting partner, RpfB, a lytic transglycosylase, have been identified as being involved in septum cleavage [14]. Although these findings provide important insights into mycobacterial cell division, major gaps in our understanding of this process still remain. The genome of *M. tuberculosis* contains multiple genes annotated as encoding cell division proteins, but their functions and the signals driving coordination of cell division with other cell cycle processes are largely unknown [14]. Several of these genes are predicted to be essential and therefore represent prospective targets for drug design. Apart from providing clues to how *M. tuberculosis* enters a latent phase, identifying and functionally annotating mycobacterial cell division genes could conceivably lead to identification of several novel anti-tuberculosis drug targets.

In this study, we identified mycobacterial (*M.smegmatis* and *M. tuberculosis*) homologues of *ftsW* and *amiC* by genetic

* Corresponding author.
E-mail addresses: sujatasharma8@gmail.com (S.S. Gaiwala Sharma), vimal@ccmb.res.in (V. Kishore), raghu@ccmb.res.in (T.R. Raghunand).
[1] Equally contributed. Tel.: +91 40 27192924; fax: +91 40 27161591.

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complementation of *E. coli* strains carrying mutations in these genes. Using gene expression studies and *M. smegmatis* strains overexpressing the identified genes, we validate their cell-division-associated functions. Our work confirms the annotation of a key divisome-associated protein and identifies a novel PG hydrolase integral to mycobacterial cell division. Our approach also demonstrates the utility of *E. coli* as an effective system for identifying mycobacterial cell division genes.

2. Materials and methods

2.1. Bacterial strains, culture conditions and plasmids

All *E. coli* cultures were grown in LB medium at 37 °C or as specified in Table 1, and on LB agar plates. *M. smegmatis* mc^2155 was grown in Middlebrook 7H9 broth (Difco) or on Middlebrook 7H10 agar (Difco) supplemented with 10% albumin-dextrose-saline (ADS), 0.2% glycerol, and 0.05% Tween 80 at 37 °C. Antibiotics were added at the following concentrations: ampicillin, 200 μg mL^{-1}, chloramphenicol, 25 μg mL^{-1}, tetracycline, 10 μg mL^{-1}, kanamycin (50 μg $mL^{-1}$for *E. coli*, 15 μg $mL^{-1}$for *M. smegmatis*), hygromycin (200 μg $mL^{-1}$for *E. coli*, 50 μg $mL^{-1}$for *M. smegmatis*) [20].

2.2. DNA techniques

All DNA manipulations, including plasmid DNA preparation, restriction endonuclease digestion, agarose gel electrophoresis, isolation and ligation of DNA fragments and *E. coli* transformation were performed as described [24]. Mycobacterial strains were transformed by electroporation.

2.3. Complementation assays

For complementation assays, ORFs corresponding to *ftsW* and *amiC* from *E. coli*, *M. smegmatis* and *M. tuberculosis* H37Rv were amplified from their respective gDNA using specific genes (Table S1) and cloned into pTrc99A [2]. Since *ftsW* is essential in *E. coli*, the *ftsW* mutant survives only in the presence of a shelter plasmid with the native *E. coli* gene. *E. coli ftsW* was initially induced from the arabinose-inducible pBAD promoter by first culturing mutant transformants in LB + 0.2% arabinose for 16 h at 37 °C (primary cultures), followed by growth LB + 0.2% arabinose (secondary cultures) to support their growth. Exponential phase cultures were harvested and washed thrice with LB to remove traces of arabinose to prevent induction of native *ftsW*. Complementation was tested by growing cultures in LB supplemented with glucose and antibiotics (Table 1) in the presence of 0.5 mM IPTG, which mediates induction of the mycobacterial *ftsW* homologs from their pTrc99A constructs. The *E. coli ftsW* mutant strain transformed with pTrc99A and pTrc99A-*E.coli ftsW* served as a negative and positive control, respectively, in these assays. Both controls underwent the exact same treatments as the test samples. Prior to performing these assays, the induction conditions were standardised based on the cognate mutant phenotypes. The IPTG concentration was kept constant at 0.5 mM and the phenotypes were microscopically observed 6 and 9 h post-induction. Since the positive and negative controls displayed clear WT and mutant phenotypes, respectively, following 9 h of induction, this induction period was chosen for complementation. Primary cultures of *E. coli* Δ*amiC*:*kan* transformants were incubated for 16 h at 30 °C and secondary cultures were induced with 0.5 mM IPTG at their exponential phases of growth; 1 mL of each of the above samples was then processed for microscopy. Expression of all complementing genes was confirmed by real-time RT-PCR as described below (data not shown). At least three biological replicates were performed.

2.4. Overexpression assays

For their overexpression, ORFs corresponding to the *M. smegmatis* homologues of *ftsW* and *amiC* were cloned into pTetO [8] using gene-specific primers (Table S1). The recombinant pTetO constructs were transformed into *M. smegmatis* along with the empty vector as the negative control. Transformant cultures were induced at exponential phase with 50 ng mL^{-1}anhydrotetracycline (AHTC) for 16 h at 37 °C. The induced cultures were then processed for CFU counts, microscopy and RNA isolation to confirm overexpression by RT-PCR. Data from three independent experiments were used to analyse the phenotypes depicted.

2.5. Microscopy

For DIC microscopy, pellets from 1 mL of *E. coli*/*M.smegmatis* cultures were harvested and resuspended in 200 μL of PBS (*E.coli*) or 200 μL of 20% Tween 80 (*M.smegmatis*). Since mycobacteria have a tendency to form clumps, 20% Tween 80 was used to generate an evenly dispersed suspension of cells; 3–5 μL of these samples were loaded onto microscope slides harbouring 1% agarose pads. The cells were visualised at 100 X under an oil immersion lens on either a Zeiss Axioimager Z1 or a Zeiss Axioplan 2 microscope. Cell numbers and lengths were measured manually.

Table 1
E. coli strains used in this study.

E. coli mutant strain	Strain genotype	Permissive conditions	Restrictive conditions	Antibiotics	Reference
Δ*ftsW*	EC1159–EC251 Δ (attϕ80)::pJC118-*gfp-ftsX ftsW::kan*/pBAD33-*ftsW* (pDSW406)	Presence of arabinose & absence of glucose, 37 °C	Presence of glucose & absence of arabinose, 37 °C	Amp, Kan, Chlor	[25]
Δ*amiC*::*kan*	*E.coli* MG1655 Δ*amiC*::*kan*	None, 30 °C	None, 30 °C	Amp, Kan	Keio collection mutant