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ChiZ levels modulate cell division process in mycobacteria

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SUMMARY

We have previously shown that expression of *chiZ* (Rv2719c), encoding a cell wall hydrolase, is upregulated in response to DNA damaging agents and exposure to cephalexin. Furthermore, increased levels of ChiZ lead to decreased viability, loss of membrane integrity and defects in FtsZ-GFP localization and cell division. We now show that ChiZ N′-terminal 110 amino acid region, containing the cell wall hydrolase activity, is sufficient to modulate FtsZ-GFP localization. Further, we found that FtsZ-GFP rings are stabilized in a *chiZ* deletion strain indicating that ChiZ activity regulates FtsZ assembly. Overexpression of *ftsZ* did not reverse the reduction in viability caused by overproduction of ChiZ indicating that ChiZ neither interacts with nor directly influences FtsZ assembly. Bacterial two-hybrid assays revealed that ChiZ interacts with FtsI and FtsQ, two other septasomal proteins, but not with FtsZ. Finally, we show that ChiZ is not required for virulence of *Mycobacterium tuberculosis* in murine macrophages and mice. Our data suggest that optimal levels and activity of the cell wall hydrolase ChiZ are required for regulated cell division in mycobacteria.

1. Introduction

Infections due to *Mycobacterium tuberculosis* are a leading cause of morbidity and mortality worldwide. *M. tuberculosis* is responsible for causing nearly 10 million new infections in the last year alone.[1] Furthermore, the already prevalent multidrug resistant and the emerging extremely drug resistant strains of *M. tuberculosis* add urgency to the efforts to accelerate the discovery of novel drugs and efficient vaccines against this deadly global pathogen. The ability of *M. tuberculosis* to shift between active and persistent phases of infection is key to its success as an enduring pathogen. Pathways necessary for multiplication and survival of the pathogen during early, active and persistent phases of infection have been the focus of a number of recent studies[2–5] and reviews.[6,7] In addition, bacterial pathways necessary for proliferation such as cell division and cell wall remodeling and their regulation are considered important for understanding the fundamentals of entry into and exit from the persistent state.[8–11]

While great strides have been made in the field of cell division in *Escherichia coli, Bacillus subtilis* and *Caulobacter crescentus*, mycobacterial cell division studies are still in their infancy. *M. tuberculosis* FtsZ, a key cell division player, shows slow polymerization and GTP hydrolysis activities in vitro and FtsZ activities appear to be regulated during intracellular growth.[12–14] Recent studies identified several proteins, some of which are novel, that interfere with FtsZ activities.[15–21] Other studies have identified proteins involved in cell wall synthesis and cell division pathways in *M. tuberculosis*.[22–25] Our previous studies characterized ChiZ, a small membrane protein, as a cell wall hydrolase that is induced upon DNA damage and intramacrophage growth and interferes with cell division.[15] These studies also showed that ChiZ overproduction led to destabilization of FtsZ-rings in vivo, but that purified ChiZ did not interfere with FtsZ assembly in vitro.[15] It is undetermined if ChiZ interacts with FtsZ and/or other cell division proteins. It is also unknown whether the loss of ChiZ affects FtsZ assembly and finally, whether ChiZ is needed for virulence of *M. tuberculosis*. The present study was designed to address the above questions. Our results suggest that ChiZ interacts with other septasomal complex proteins and that optimal levels of ChiZ modulate normal progression of cell division in *M. tuberculosis*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli Top 10 strain was used for cloning purposes and propagated in Luria Bertani (LB) broth or LB agar plates. Recombinant

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E. coli strains were grown in LB broth or LB agar supplemented with appropriate antibiotics as needed with kanamycin (Km) at 50 μg/ml, ampicillin (Amp) at 100 μg/ml and hygromycin (Hyg) at 10 μg/ml. *Mycobacterium smegmatis* and *M. tuberculosis* were propagated in Middlebrook 7H10 agar or broth supplemented with oleic acid, albumin, dextrose and sodium chloride (OADC). Transformants were selected on the same media supplemented with Km (15 μg/ml) or Hyg (10 μg/ml). Growth was monitored by measuring A600 of broth-grown cultures. For viability determinations, dilutions of exponential cultures were plated on 7H10 agar plates containing appropriate antibiotics and plates incubated for 3–5 days for *M. smegmatis* and ~ 3 weeks for *M. tuberculosis*. Colonies were counted and data plotted using Excel.

2.2. Molecular cloning and construction of recombinant strains

Polymerase chain reaction using deep vent or Phusion polymerases was performed to amplify various genomic DNA fragments. These full length or truncated coding regions were subsequently cloned into appropriate vectors to create the recombinant plasmids shown in Table 1. Restriction cloning sites were always included in the oligonucleotide primers used for PCR amplification. PCR products were digested with the appropriate restriction enzymes and cloned into corresponding sites of the desired vectors (see Table 1). All cloned fragments were confirmed by sequencing. Full length $ChiZ_{smeg}$ (MSMEG_2742) or its various truncations were cloned following PCR amplification of the appropriate regions (Table 1). Recombinant plasmids were used to transform *M. smegmatis* and *M. tuberculosis* as described before[26] and transformants were selected on 7H10 agar plates containing appropriate antibiotics. Recombinant strains were confirmed by recovery of plasmids by bead-beating followed by restriction analysis or PCR as described.[26]

2.3. Microscopy

For microscopy, wild type and recombinant *M. smegmatis* and *M. tuberculosis* strains were grown to exponential phase, cells pelleted by centrifugation at 3000x g and resuspended in PBS. GFP-FtsI could not be visualized in *M. tuberculosis* due to bleaching of fluorescent signal from paraformaldehyde fixation.[19] Therefore, some fusion protein localization experiments were carried out in the surrogate host, *M. smegmatis*.[19] The cells were visualized by brightfield and fluorescence microscopy using a Nikon Eclipse E600 microscope equipped with a CoolSnap ES CCDcamera (Photometrics) and a high-pressure mercury lamp (Nikon). A Nikon B2A filter set (Ex450–490/Em515) was used for GFP strains. The YFP and mCherry fusion were imaged using a Nikon YFP filter (Ex426–446/Em460–500) and a mCherry filter (Ex560/Em630) set from Chroma, respectively. Images were analyzed using MetaMorph 6.2 software (Universal Imaging Corporation). At least 100 cells from each set were scored for localization patterns and cell length measurements. Images were optimized using Adobe Photoshop CS4.

Table 1
Strains and plasmids used in the study.

Name	Description	Reference
Strains		
Top10F′	*Escherichia coli* strains	Invitrogen
H37Rv	Virulent wild type *Mycobacterium tuberculosis*	Laboratory stock
mC^2155	*Mycobacterium smegmatis*	Laboratory stock
$\Delta chiZ_{smeg}$	*M. smegmatis chiZ* deletion strain	[15]
$\Delta chiZ_{TB}$	*M. tuberculosis chiZ* deletion strain	[15]
Cloning vectors		
pJAM2	*E. coli-Mycobacterium* shuttle vector with *amidase* promoter, replicating, Km^r	[37]
pMG103	*E. coli-Mycobacterium* shuttle vector with *amidase* promoter, integrating, Km^r	[18]
pLR56	*E. coli – Mycobacterium* shuttle vector, integrating, with *tet* promoter and tet repressor; Km^r	[18]
pMV306	L5 integration vector, Hyg^r	MedImmune Inc.
pKT25	*E. coli* expression vector allowing fusions to C-terminal of the T25 fragment of cyaA, Km^r	[27]
pUT18C	*E. coli* expression vector allowing fusions to C-terminal of the T18 fragment of cyaA, Amp^r	[27]
pKNT25	*E. coli* expression vector allowing fusions to N-terminal of the T25 fragment of cyaA, Km^r	[27]
Plasmids used in this study*		
pPP79	*gfp-*$FtsI_{TB}$ cloned in pMG103, Km^r	[19]
pRR13	*Pami::*$ftsZ_{smeg}$*-gfp in pJFR19*	[15]
pRD3	*Ptet::*$ftsZ_{TB}$*-gfp in pLR56*	[18]
pKNT25ftsZ	$ftsZ_{TB}$ cloned as C-terminal fusion into pKNT25 low copy replicating, Km^r	[18]
pUT18ftsZ	$ftsZ_{TB}$ cloned as C-terminal fusion into pUT18 high copy replicating vector, Amp^r	[18]
pRD60	*wag31* cloned as N-terminal fusion into pUT18C vector, Amp^r	This study
pRD64	*wag31* cloned as N-terminal fusion into pKT25 vector, Km^r	This study
pRD76	*ftsW* cloned in pUT18, Amp^r	This study
pRD77	*ftsW* cloned in pKT25, Km^r	This study
pMK18	$ftsI_{TB}$ cloned in pUT18C, Amp^r	[18]
pMK19	$ftsI_{TB}$ cloned in pKT25, Km^r	[18]
pMK20	$ftsQ_{TB}$ cloned in pUT18C, Amp^r	[19]
pMK21	$ftsQ_{TB}$ cloned in pKT25, Km^r	[19]
pLR54	*PdnaA::*$chiZ_{smeg}\Delta LysM$	[15]
pLR55	*chiZ*Δ*N-term encoding* $ChiZ_{smeg}$ *aa 91-end,* cloned under *Pami* in pJAM2	This study
pJFR78	*Pami::*$ftsZ_{smeg}$ cloned in pMV306	[18]
pJFR84	*gfp-*$ftsQ_{smeg}$ cloned in pJAM2, Km^r	[18]
pACR9	$chiZ_{L\psi\sigma M}$ encoding $ChiZ_{smeg}$ aa 105 to end, cloned under *Pami* in pJAM2	This study
pACR24	$chiZ_{N\text{-}term}$ encoding $ChiZ_{smeg}$ aa 1–90, cloned under *Pami* in pJAM2	This study
pSAR58	*PdnaA::*$chiZ_{smeg}$	[15]

* Sequences of primers used for PCR amplification of cloned inserts are available upon request.