SUPPLEMENTAL MATERIAL

SUPPLEMENTARY METHODS

Construction of *ftsI* conditional expression strain. Unmarked deletion in the *ftsI* gene was delivered through a suicide recombination substrate, pMP15 (Table S1), using a two-step recombination protocol (1). pMP15 was created in three steps. First, the 5' end of the *ftsI* gene (18 bp-*ftsI*) and its upstream region were amplified using the primer pair MsFtsIGR1/MsFtsIGR2 and cloned into *Pst*I-*Hind*III sites of p2NIL to create pMP12. Next, the 3' end of the *ftsI* gene (929 bp) and its downstream region were amplified using the primer pair MsFtsIGR3/MsFtsIGR4 and cloned into *Hind*III-*Pac*I sites of pMP12 to create pMP14. Finally, a 6-kb *Pac*I marker cassette from pGOAL17 carrying *lacZ* and *sacB* genes was cloned into pMP14 to create the pMP15 for the subsequent selection of single crossover (SCO) recombinants, which were blue, Km^r and sucrose sensitive, as previously described (2). Southern hybridization was performed to confirm the SCOs (Fig. S2). Next, pMP16 plasmid expressing *Pami::ftsI* was integrated into SCO strain to create SCO-P_{ami}*ftsI*. The SCO and SCO-P_{ami}*FtsI* strains were processed to select for double crossovers (DCO) that were white, Km^S and resistant to sucrose. PCR and Southern hybridization were used to distinguish between the WT and mutant DCOs. Only WT DCO colonies were obtained with SCO, whereas both WT and mutant DCO colonies were obtained with SCO-P_{ami}*FtsI* (data not shown). Thus, as in other bacteria, *ftsI* is essential in *M. smegmatis* and was only deleted in the presence of another copy of the gene.

SUPPLEMENTARY DATA

FIG S1

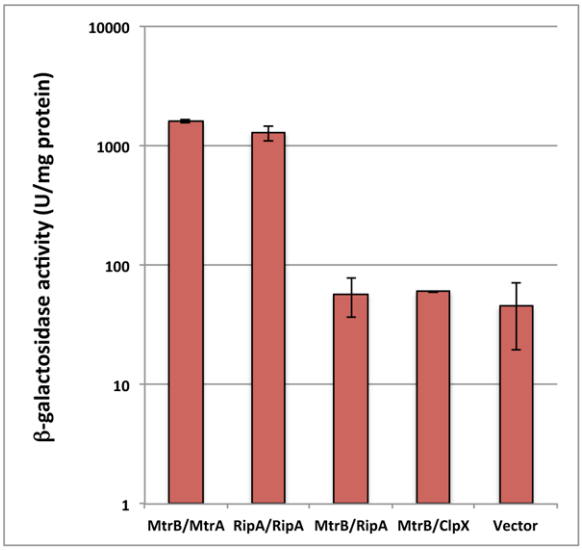

FIG S1 MtrB interactions. Interactions of MtrB with RipA and ClpX proteins were examined using BACTH assays as described (3). Indicated combinations of various BACTH expression vectors (Table S1) were used to transform *E. coli* BTH101 and transformants were processed for measurement of β-galactosidase activity. MtrB/MtrA and RipA/RipA are shown as positive controls and MtrB/empty vector (vector) as negative control. Data shown are means ± standard deviation from 3 independent experiments.

FIG S2

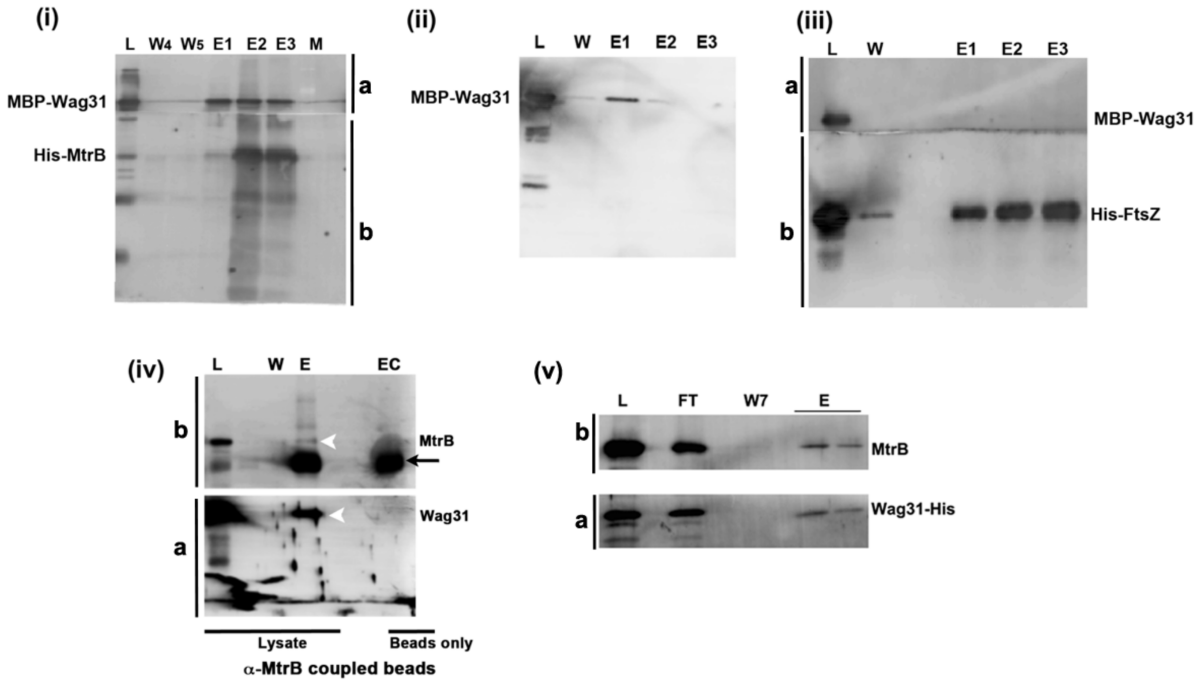

FIG S2 MtrB-Wag31 interactions. Interactions of MtrB with Wag31 were analyzed by pull-down (i – iii, v) or co-IP (iv) assays. Experimental details and figure descriptions are as described in materials and methods and in legends for Fig. 1C separately. Lysate or load used for pull-down or IP (L), wash (W) and elution (E) samples were separated by SDS-PAGE, transferred to PVDF membrane and proteins identified by immunoblotting with specific antibodies. Panels 'i' to 'iii' are full gel images for data in Fig. 1C (i – iii). Lanes L – load, W – last of 5 washes, W4, W5 – washes 4 and 5. E1, E2 and E3 are elutions with buffer containing 100 mM, 300 mM and 1 M imidazole, respectively. Panel iv – Co-immunoprecipitation of Wag31 with MtrB from lysates of *M.*

smegmatis Ptet::mtrB strain using α-MtrB coupled BioMag Plus Amine beads. White arrowheads – MtrB and Wag31 bands. Black arrow – IgG band. (v) Pull-down of MtrB with Wag31-His from *M. smegmatis* lysate. Pull-down assays were performed with lysates prepared from *M. smegmatis Pami::wag31-His$_{10}$* (Table S1) using the Profound Pull-down assay kit (Pierce) as per manufacturer's instructions. L (load), flow-through (FT), last wash or wash 7 (W7) and elution (E) fractions were separated by SDS-PAGE and proteins visualized by immunoblotting with Wag31 and MtrB specific antibodies. As needed, for all experiments, the blots were cut and top and bottom halves probed with indicated antibodies. **a** – probed with α-Wag31 or α-MBP; **b** – probed with α-MtrB or a-FtsZ.

Fig. S3

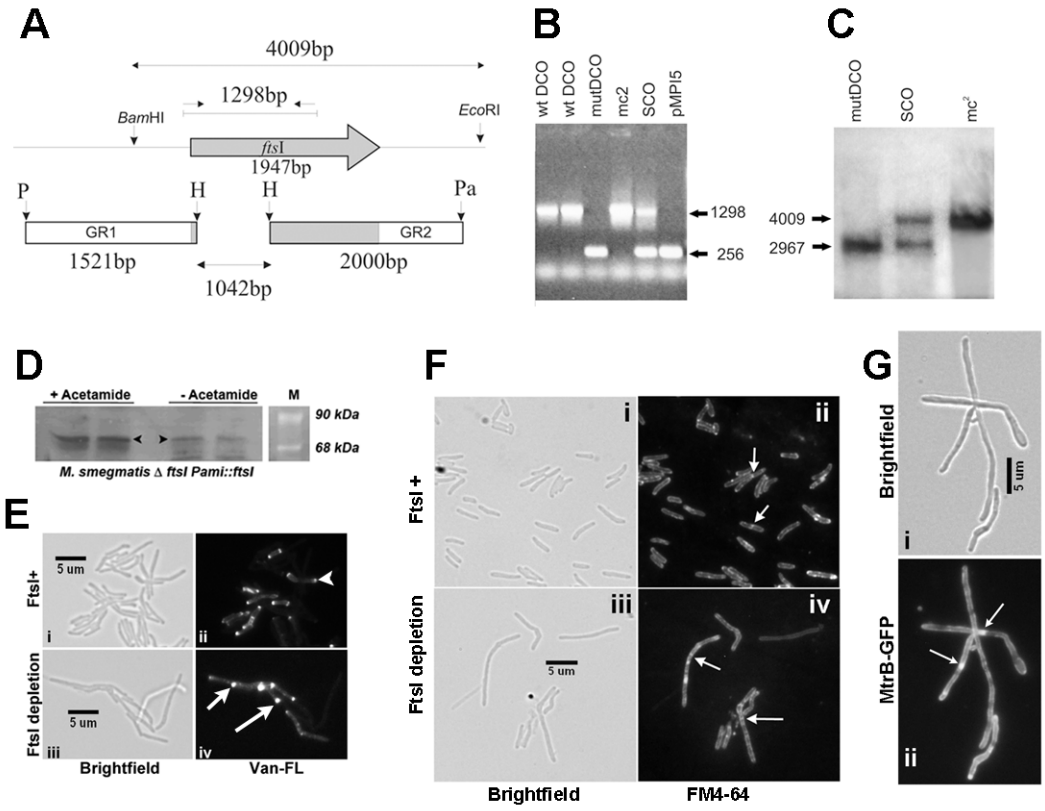

FIG S3 Construction and characterization of *ftsI* deletion/ conditional expression strain. (A) Cartoon of *M. smegmatis ftsI* (grey arrow) region. The *Eco*RI and *BamHI* recognition sites are denoted. Shown below are the GR1 and GR2 fragments along with their sizes used for creating pMP12 and pMP14 plasmids (Table S1). 1042-bp represents the size of the *fts*I gene deleted. P,H and Pa denote *Pst*I, *Hind*III and *Pac*I restriction enzyme recognition sites, respectively. (B) Agarose gel showing PCR amplified fragments from various strains. Lanes: wt DCO, WT double cross-over (DCO); mutDCO, mutant DCO; mc^2, chromosomal DNA isolated from WT *M. smegmatis*; SCO, single cross-over and pMP15, plasmid DNA. The DNA fragments corresponding to WT *ftsI* (1298 bp) or Δ *ftsI* (256 bp) are marked by black arrows. (C) Southern hybridization profiles of genomic DNA isolated from WT *M. smegmatis* (mc^2), SCO and mutDCO. *Eco*RI and *Bam*HI enzyme digested DNA was resolved in 1% agarose gel, processed for Southern and probed with *fts*I gene fragment amplified using MsFtsIs and MsFtsIr primers and pMP15 as a template. The expected hybridization bands of 4009 bp for WT and 2967 bp for mutant patterns are indicated by arrows. (D-G) Consequences of FtsI depletion. *M. smegmatis* Δ *ftsI Pami::ftsI* strain was grown overnight in 7H9 broth containing 0.2% acetamide, washed twice in acetamide-free media and then grown without or with acetamide for 14 hours. Cells were harvested and lysed by beating as described (3), ten microgram of total cell lysates separated by SDS-PAGE and FtsI levels were determined by immunoblotting using α-FtsI as described in text (D). Black arrowhead – FtsI. (E) Cells were stained with Van-FL and examined by brightfield and fluorescence microscopy. Note septal (arrows) and polar (arrowhead) staining with Van-FL. (F) *ftsI* was strain grown without or with 0.2%

acetamide for 10 h, stained with membrane staining dye FM4-64 as described (4) and visualized by brightfield and fluorescent microscopy. Arrows – septal staining. (G) *M. smegmatis* Δ *ftsI Pami::ftsI* expressing *Ptet::mtrB-gfp* was grown without acetamide for 14 hours as described under Fig. 2C and cells examined by brightfield and fluorescence microscopy. Arrow – diffuse septal MtrB-GFP bands.

FIG S4

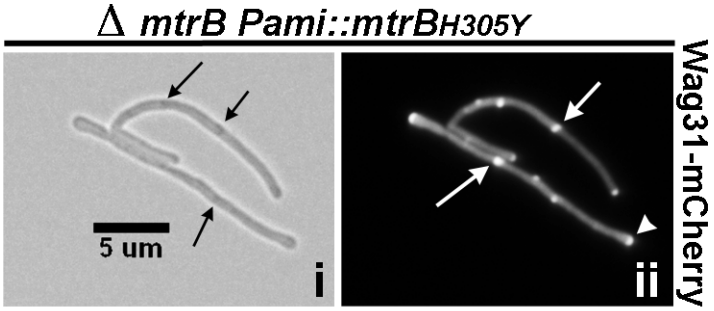

FIG S4 Characterization of Δ *mtr*B strain. (A) Localization of Wag31-mCherry. *M. smegmatis* Δ *mtr*B *Pami::mtr*B_{H305Y} expressing *Ptet::wag*31-*mCherry* was grown with 0.2% acetamide for 6 h and anhydrotetracycline at 50 ng/ml was added 1 hour prior to processing samples for microscopy. Brightfield (i) and fluorescence (ii) microscopy was carried out as described (3). Note Wag31-mCherry fluorescent bands at poles and at septa in panel ii. Black arrow – septa; white arrow and arrowhead – septal and polar Wag31-mCherry localizations, respectively.

FIG S5

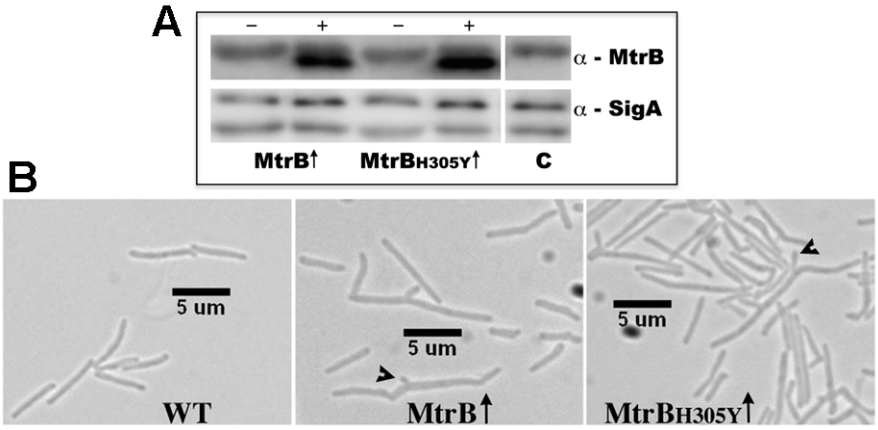

FIG S5 Characterization of *mtr*B strains: (A). Intracellular levels of MtrB and SigA (loading control) in *M. smegmatis* overproducing either WT MtrB (MtrB↑) or mutant $MtrB_{H305Y}$ ($MtrB_{H305Y}$↑) (see also Table S1) were monitored by immunoblotting and quantitated using QuantityOne software (3). - and + indicate cultures grown without and with 50 ng/ml anhydrotetracycline for 6 hours. C – WT *M. smegmatis*. α-SigA antibodies pick up 2 bands in *M. smegmatis* (5) (B) *M. smegmatis Ptet::mtrB* or *Ptet::mtrB*$_{H305Y}$ cultures were induced with 50 ng/ml anhydrotetracycline for 6 hours and cell morphology examined by brightfield microscopy. *M. smegmatis* WT is shown as control. Arrowheads indicate swollen poles or bud-like structures.

TABLE S1

Name	*Description*	*Reference*
STRAINS		
Top10F'	*Escherichia coli* strain for cloning	Invitrogen
BL21 (DE3) pLysS	*E. coli* expression strain	Novagen
BTH101	*E. coli* bacterial two-hybrid host strain	Euromedex
mC²155	*M. smegmatis*	Laboratory stock
FZ3	*M. smegmatis ftsZ* conditional expression strain	(6)
D *mtrB*	*M. smegmatis mtrB* deletion strain strain	(3)
D *wag31::Pami-wag3*	*M. smegmatis wag31* conditional expressi strain	(7)
D *ftsI::Pami-ftsI*	*M. smegmatis ftsI* conditional expression strain	This study
CLONING VECTORS		
pLR56	*E. coli – Mycobacterium* shuttle vect integrating, with tet promoter and tet repress Km^r	(8)
pLR52	*E. coli – Mycobacterium* shuttle vect replicating, with tet promoter and tet repress Hyg^r	(8)
pMG103	*E. coli – Mycobacterium* shuttle vect replicating, with amidase promoter, Km^r	(8)
pKT25	*E. coli* expression vector allowing fusions to terminal of the T25 fragment of *cyaA* , Km^r	(9)
pUT18C	*E. coli* expression vector allowing fusions to terminal of the T18 fragment of *cyaA*, Amp^r	(9)
pKNT25	*E. coli* expression vector allowing fusions to terminal of the T25 fragment of *cyaA* , Km^r	(9)
pUT18	*E. coli* expression vector allowing fusions to terminal of the T18 fragment of *cyaA*, Amp^r	(9)
p2NIL	Non-replicating recombination vector, Km^r	(1)
pGOAL17	Plasmid carrying PacI cassette, Amp^r	(1)

PLASMIDS USED IN THIS STUDY		
pEB16	*Wag31-mCherry* cloned in pMG103 vector, Km^r	(5)
pEB25	*wag31$_{TB}$* cloned as a fusion to gene for malto binding protein in pMALc4e, Amp^r	This study
pPP79	*gfp-FtsI* cloned in pMG103 vector, Km^r	(4)
pSAR1	*ftsZ$_{TB}$* cloned in pET19b vector, Amp^r	(10)
pSVM4	*mtrB$_{TB}$* cloned into pLR52 vector, Hyg^r	This study

pRD20	*mtrB$_{sol}$ (CDS 234 to end)* cloned in pET19b vect Amp^r	This study
pJFR79	*ftsZ*$_{TB}$-*gfp* under the *Pami* promoter in pJAM2, Km^r	(10)
pRD73	*mtrB$_{TB}$-gfp* cloned in pLR52 vector, Hyg^r	(5)
pRD60	*wag31* cloned as N-terminal fusion in pUT18C, Amp^r	(11)
pRD64	*wag31* cloned as N-terminal fusion in pKT25 vector, Km^r	(11)
pRD83	*mtrB$_{H305Y}$* cloned in pLR56 vector, Km^r	(3)
pRD101	*mtrB$_{H305Y}$* cloned as C-terminal fusion into pKNT25 vector, Km^r	This study
pRD102	*mtrB$_{TB}$* cloned in pJFR19 vector (*Pami*::*mtr* Hyg^r	(3)
pRD104	*mtrB$_{H305Y-TB}$* cloned in pJFR19 vect (*Pami*::*mtrB$_{H305Y}$*), Hyg^r	(3)
pDS4	*mtrA$_{Y102C}$* under *Pami* in pJFR19, Hyg^r	(3)
pKS23	*mtrB$_{H305Y}$* cloned as C-terminal fusion into pUT vector, Amp^r	This study
pKS6	*mtrB$_{TB}$* cloned as C-terminal fusion into pUT vector, Amp^r	This study
pKS7	*mtrB$_{TB}$* cloned as C-terminal fusion into pKNT vector, Km^r	This study
pKS12	*ripA$_{TB}$* cloned as N-terminal fusion into pUT1 high copy replicating vector, Amp^r	This study
pKS13	*ripA$_{TB}$* cloned as N-terminal fusion into pKT low copy, replicating vector, Km^r	This study
pKS74	*Wag31-His$_{10}$* cloned in pMG103, Km^r	This study
pMK18	*ftsI$_{TB}$* cloned in pUT18C, Amp^r	(8)
pMK19	*ftsI$_{TB}$* cloned in pKT25, Km^r	(8)
pPknA-T25	*pknA$_{TB}$* cloned in pKT25 vector, Km^r	This study
pPknA-T18C	*pknA$_{TB}$* cloned in pUT18c, Amp^r	This study
pPknB-T25	*pknB$_{TB}$* cloned in pKT25 vector, Km^r	This study
pPknB-T18C	*pknA$_{TB}$* cloned in pUT18c, Amp^r	This study
pMP12	1521-bp *Pst*I-*Hind*III fragment including 5' end *fts*I (18 bp) and its upstream region cloned p2NIL, Km^r	This study
pMPI4	2000 bp *Hind*III-*Pac*I fragment including 3' e of *fts*I (929 bp) and its downstream region	This study

	pMPI2, Km^r	
pMPI5	pMPI4 with *Pac*I cassette from pGoal17, Km^r	This study
pMPI6	*ftsIsmeg* CDS under *Pami* in pMV306, Hyg^r	This study

TABLE S2. Oligonucleotides used for cloning

Name of primer	Sequence (5' -> 3')	Construct created
MVM883	Agaaccttaattaagagccccaccagggaggaagccgaacgat gatcttcggctcgcgccg	pSVM4, pRD83
MVM884	atcggatttaaattcaaccgctccactccgcg	pSVM4, pRD83
MVM830	tctagaggatcccatgccgcttacacctgccga	pRD60, pRD64
MVM831	ttacttaggtacccggtttttgccccggttgaattgatcga	pRD60, pRD64
MVM846	ttgctctagagatgatcttcggctcgcgccg	pKS6, pKS7, pKS23, pRD101
MVM847	ttacttaggtacccgaccgctccactccgcgtgct	pKS6, pKS7, pKS23, pRD101
MVM919	cgcggatccctagtttttgccccggttgaatt	pEB30
MVM920	gggaattccatatgccgcttacacctgccga	pEB16, pKS74
MVM923	cacagtactgtttttgccccggttgaatt	pEB16
wag31F	agaggatcccatggccgcgggcggcggtgccgg	pRD60, pRD64
wag31R	ttacttaggtacccgcgccgagaccgccgccggcg	pRD60, pRD64
MsFtsIGR1	aactgcagcgcggcgcaccggcggccatcc	pMP12
MsFtsIGR2	ccaagcttgtcgccccgccggctcatgcg	pMP12
MsFtsIGR3	ccaagcttggcagatgggcaatccgtcgg	pMP14
MsFtsIGR4	ccttaattaaggcgacaccccgaccgcgtcg	pMP14
MsFtsIs	cgggatccatgagccggcggggcgaccg	pMP16
MsFtsIr	gctctagattaggtggcctgcaatgtcaac	pMP16
Wag31_R_PacI	ccttaattaactagtttttgccccggttg	pKS74

TABLE S3. Oligonucleotide primers used in qRT-PCR assays

Target/gene Primer Name Sequence (5' -> 3')		
Target	Gene Primer Name	Sequence (5'->3')
sigA	sigAqRT_F	gtg ggc agc gac caa agc aag
	sigAqRT_R	act tcg ccg ctg ttc gct tg
mtrA	MR199-Msmg-MtrA_F	tca ctg gcc gag atg ctc ac
	MR199-Msmg-MtrA_R	cac atc gat gcc gtt cat cc
mtrB	Msmg-mtrBqRT1	tgc aga ccg aag ggt tct cc
	Msmg-mtrBqRT2	gcg cga tgg tgc tct cct cg
wag31	wag31-Msmeg-qRT1	ttc cga gat cat ggg aac cat tg
	wag31-Msmeg-qRT2	gag ctg cga ctc cag gta ggt c
ftsI	MR197-Msmg-FtsIF	ccg acg gtt cgg tga cct ac
	MR197-Msmg-FtsIR	ctt cgc cat ctg gac ctg c
dnaA	dnaA-Msmeg-qRT1	ctt cat caa ctc gct gcg tga c
	dnaA-Msmeg-qRT2	tgg aag aac tcc tcc tgg atg c
ripA	ripA-MsmgqRT1	gtt cct gca gaa gct cgg aat c
	ripA-MsmgqRT2	cac gag tag ggc aca ccg atc
fbpB	MS85BqRT1	ttc gag atg ttc ctc gac tc
	MS85BqRT2	cca ctt gta ggt gac gca g

REFERENCES

1. **Parish T, Stoker NG.** 2000. Use of a flexible cassette method to generate a double unmarked Mycobacterium tuberculosis tlyA plcABC mutant by gene replacement. Microbiology **146 (Pt 8):**1969-1975.
2. **Chauhan A, Madiraju MV, Fol M, Lofton H, Maloney E, Reynolds R, Rajagopalan M.** 2006. Mycobacterium tuberculosis cells growing in macrophages are filamentous and deficient in FtsZ rings. Journal of bacteriology **188:**1856-1865.
3. **Plocinska R, Purushotham G, Sarva K, Vadrevu IS, Pandeeti EV, Arora N, Plocinski P, Madiraju MV, Rajagopalan M.** 2012. Septal Localization of the Mycobacterium tuberculosis MtrB Sensor Kinase Promotes MtrA Regulon Expression. The Journal of biological chemistry **287:**23887-23899.
4. **Plocinski P, Ziolkiewicz M, Kiran M, Vadrevu SI, Nguyen HB, Hugonnet J, Veckerle C, Arthur M, Dziadek J, Cross TA, Madiraju M, Rajagopalan M.** 2011. Characterization of CrgA, a new partner of the Mycobacterium tuberculosis peptidoglycan polymerization complexes. Journal of bacteriology **193:**3246-3256.
5. **Plocinski P, Arora N, Sarva K, Blaszczyk E, Qin H, Das N, Plocinska R, Ziolkiewicz M, Dziadek J, Kiran M, Gorla P, Cross TA, Madiraju M, Rajagopalan M.** 2012. Mycobacterium tuberculosis CwsA interacts with CrgA and Wag31 and the CrgA-CwsA complex is involved in peptidoglycan synthesis and cell shape determination. Journal of bacteriology.
6. **Dziadek J, Rutherford SA, Madiraju MV, Atkinson MA, Rajagopalan M.** 2003. Conditional expression of Mycobacterium smegmatis ftsZ, an essential cell division gene. Microbiology **149:**1593-1603.
7. **Kang CM, Nyayapathy S, Lee JY, Suh JW, Husson RN.** 2008. Wag31, a homologue of the cell division protein DivIVA, regulates growth, morphology and polar cell wall synthesis in mycobacteria. Microbiology **154:**725-735.
8. **Dziedzic R, Kiran M, Plocinski P, Ziolkiewicz M, Brzostek A, Moomey M, Vadrevu IS, Dziadek J, Madiraju M, Rajagopalan M.** 2010. Mycobacterium tuberculosis ClpX interacts with FtsZ and interferes with FtsZ assembly. PloS one **5:**1-14.
9. **Karimova G, Dautin N, Ladant D.** 2005. Interaction network among Escherichia coli membrane proteins involved in cell division as revealed by bacterial two-hybrid analysis. Journal of bacteriology **187:**2233-2243.
10. **Rajagopalan M, Atkinson MA, Lofton H, Chauhan A, Madiraju MV.** 2005. Mutations in the GTP-binding and synergy loop domains of Mycobacterium tuberculosis ftsZ compromise its function in vitro and in vivo. Biochem Biophys Res Commun **331:**1171-1177.
11. **Vadrevu IS, Lofton H, Sarva K, Blasczyk E, Plocinska R, Chinnaswamy J, Madiraju M, Rajagopalan M.** 2011. ChiZ levels modulate cell division process in mycobacteria. Tuberculosis (Edinb) **91 Suppl 1:**S128-135.