Published in final edited form as:

Tuberculosis (Edinb). 2011 December ; 91S1: S150–S155. doi:10.1016/j.tube.2011.10.025.

Localization of acidic phospholipid cardiolipin and DnaA in mycobacteria

Erin Maloney, Sai Chandana Madiraju, Malini Rajagopalan, and Murty Madiraju*

Biomedical Research, The University of Texas Health Science Center, 11937 U.S. Hwy @271, Tyler, TX- 75708-3154

Abstract

Acidic phospholipids such as cardiolipin (CL) have been shown to modulate *Mycobacterium tuberculosis* (*Mtb*) DnaA interactions with ATP. In the present study, using nonyl acridine orange fluorescent dye we localized CL-enriched regions to midcell septa and poles of actively dividing cells. We also found that CL-enriched regions were not visualized in cells defective for septa formation as a consequence of altered FtsZ levels. Using *Mtb* cultures synchronized for DNA replication we show that CL localization could be used as a marker for cell division and cell cycle progression. Finally, we show that the localization pattern of the DnaA-green fluorescent fusion protein is similar to CL. Our results suggest that DnaA colocalizes with CL during cell cycle progression.

1. Introduction

Mycobacterium tuberculosis (*Mtb*) is a slow-growing pathogenic bacterium and the causative agent of the infectious disease tuberculosis. This bacterium has a doubling time of ~ 24 hours, and the pathogen's efficiency as an infectious agent relies in part on its ability to shift from an active growth state to a non-replicative persistent state within the host. *Mtb* in this persistent state maintains metabolic activity¹ and presumably, favorable conditions resulting in reactivation of the bacterium can lead to active infection². Such a shift between the two growth states requires tight regulation of cell cycle mechanisms; of particular importance are mechanisms governing initiation of DNA replication.

DnaA protein is the initiator of DNA replication and binds to specific nucleotide sequences within the chromosomal origin of replication or *oriC*^{3–5}. Studies with *Escherichia coli* DnaA revealed that it binds ATP and ADP with equal affinity, and hydrolyzes ATP. The hydrolytic product ADP associates tightly with DnaA and the ADP-bound form of DnaA is not active for *oriC* replication; acidic phospholipids promote dissociation of nucleotides and activation of DnaA⁶. Binding of ATP-bound form of DnaA to the *oriC* results in opening of the duplex, subsequent assembly of other components and DNA synthesis^{7–10}. The DnaA protein of *Mtb* is essential, has been shown to bind and hydrolyze ATP, and bind to *oriC*^{11–16}. *Mtb* DnaA forms an oligomeric complex on the origin, mediated by its ATPase activity¹⁵ and this is followed by strand melting¹⁷. *Mtb* DnaA protein associates with

© 2011 Elsevier Ltd. All rights reserved.

*Address for correspondence: murty.madiraju@uthct.edu.

Competing interests: The authors have no conflicts of interest to declare.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

membrane lipids¹³, and like the *E. coli* counterpart, its interactions with ATP and ADP are also modulated by membrane lipids¹². However, the mechanism(s) regulating the replication initiation process in *Mtb* are largely unknown.

Our earlier studies using 10-nonyl acridine orange (NAO), a fluorescent dye, determined that CL-enriched regions are confined to septa and cell poles of actively growing *M. smegmatis* and *Mtb*¹⁸. These are nascent growth zones in mycobacteria^{18, 19}. Independently, we showed that *lysX* mutant deficient in production of lysinylated phosphatidylglycerol is elongated, defective for cell division and exhibits altered NAO staining pattern¹⁸. We infer from these results that NAO, which stains CL, could be used as a marker for evaluating the status of cell division in mycobacteria. Given the affects of membrane phospholipids on DnaA activity, it is also assumed, but not proven that *Mtb* DnaA interacts with acidic phospholipids in vivo. The present study is undertaken to address two independent questions: (1) whether NAO staining could be used to mark septa and cell division status of mycobacteria; (2) whether the in vivo localization of DnaA and CL are similar.

2. Materials and Methods

2.1. Growth and storage conditions for bacterial strains

E. coli strains were grown in Luria-Bertani (LB) broth or agar media. *M. smegmatis* MC²155 and *M. tuberculosis* H37Rv strains were grown in Middlebrook 7H9 broth supplemented with albumin (5 g/L) and dextrose (2 g/L); in addition, *Mtb* cultures were supplemented with oleic acid (60 µL/L) and catalase (3 mg/L). *E. coli* recombinant strains were grown with ampicillin (amp, 50 µg/mL) or kanamycin (km, 50 µg/mL) or hygromycin (hyg, 200 µg/mL). *M. smegmatis* and *Mtb* strains were grown in media supplemented with 25 µg/mL km or 10 µg/mL hyg. Growth was monitored by reading the optical density at 600 nm. Acetamide, when required was used at a final concentration of 0.2%. *M. smegmatis* FtsZ-72 is a *ftsZ* merodiploid strain expressing *ftsZ* from inducible amidase promoter (*Pami*) integrated at *attB* locus²⁰. Addition of acetamide for 6 h leads to a 6-fold increase in FtsZ levels in this strain. *M. smegmatis* FZ3-78 (also referred to as Δ *ftsZ*, *Pami::ftsZ*) is the *ftsZ* conditional expression strain which requires acetamide for normal cell division and viability²¹ and removal of acetamide results in the depletion of intracellular FtsZ levels. *Mtb dnaAcos115* is a cold-sensitive *dnaA* strain²². In this strain, the native *dnaA* was deleted and was replaced by an integrated copy of *dnaA* carrying 3 mutations at codons 73, 151 and 215. The strain exhibits a cold sensitive phenotype and has been characterized²².

2.2. Molecular cloning and construction of recombinant strains

M. smegmatis strains for overproduction and depletion of FtsZ were previously described²¹. For expression of *gfp-dnaA* fusion, the *Mtb dnaA* gene was amplified using oligonucleotide primers, SK3 (5'-CGGGATCCTTGACCGATGACCCG-3') and MVM379 (5'-TGTTCTAGACTACTAGCGCTTGGAGCGCTGAC-3') and cloned into the pGEM-T-easy vector. A 700-bp *gfp* coding region was amplified using primers MVM469 (5'-GCGACCAGTACTAAAGGAGAAGAACTTTTCACT-3') and MVM187 (5'-CGGGATCCCTGCAGGTTGTTGTTTTGTATAGTTCATCC-3') and cloned at the 5' end of the *dnaA* gene in pGEM-T-easy. The entire *gfp:dnaA* fragment was released, placed downstream of the *Pami* in pMG103 (km^r)²³. This construct was used to electro-transform *M. smegmatis* and *Mtb* competent cell, and km^r transformants were screened by microscopy for observation of fluorescence (see below). To create strains with *gfp-dnaA* as the sole source of *dnaA*, the same construct was also used to swap the functional copy of integrated *dnaA* (*Pami::dnaA*, hyg^r) in the previously constructed *M. smegmatis dnaA* conditional knockout strain²⁴. Presences of colonies on km containing agar media plates indicate the

swapping of the resident *Pami::dnaA* (hyg^r) plasmid with *Pami::gfp-dnaA* (km^r). The km^r resistant colonies were further purified, confirmed by PCR followed by sequencing. Exponential culture of *M. smegmatis* Δ *dnaA*, *Pami::gfp-dnaA* strain was grown with 0.2% acetamide for various time points and cells examined by fluorescence microscopy (see below).

2.3. Cell staining techniques and fluorescence microscopy

Use of NAO for staining CL enriched regions of *Mtb* was previously described¹⁸. Briefly, NAO was added during growth of cultures for either 1 h (for *M. smegmatis*) or 24 h (for *Mtb*) and cells harvested for microscopy. *M. smegmatis* cells were pelleted and suspended in fresh media prior to microscopy. *Mtb* pellets were suspended in 4% para-formaldehyde and stored at 4°C for 24 h before imaging. A Nikon Eclipse E600 microscope with an attached CoolSnap ES CCD camera (Photometrics) and high-pressure mercury lamp (Nikon) was used. A Nikon B2A filter set (Ex450-490/Em515) was used for GFP. NAO-stained cells were imaged using a custom NAO filter set (Ex450-490/Em645, Chroma Technology) for red fluorescence and the Nikon B2A filter set for green fluorescence. Images were analyzed using MetaMorph 6.2 software (Universal Imaging Corporation) and optimized using Adobe Photoshop 7.0.

2.4. Statistical calculations

Probability of data correlation was calculated using the Chi square test and P values of comparisons were calculated using Student's T test.

3. Results

3.1. NAO staining of *M. smegmatis* strains defective for cell division

To begin testing whether NAO staining could be used as a marker of cell division, we stained *M. smegmatis* and *Mtb* cells at different stages of growth with NAO. It is known that NAO binds to CL in membranes of mitochondria at 2: 1 ratio and creates a stacked pair that is dimer-like. This dimerization results in a shift in absorbance from 495nm to 474nm, which is accompanied by a shift in the emission from green (525nm) to red (640nm). Thus, binding of NAO to CL, but not to other acidic phospholipids, promotes a green to red shift in fluorescence [reviewed in¹⁸]. Consistent with earlier published data, we found NAO stained regions at septa and poles of exponentially growing *M. smegmatis* (Fig. 1) and *Mtb* cells (data not shown)¹⁸. However, the stationary phase cultures showed poor staining (Fig. 1). Next, to understand the correlation between NAO enriched regions and cell division, we stained *M. smegmatis* FtsZ-72 cells grown with 0.2% acetamide for 6 and 18 h and visualized by microscopy. FtsZ protein is the initiator of cell division and localizes at midcell sites as a ring called the Z-ring²⁵; overproduction or conditional depletion of FtsZ levels interfere with Z-ring assembly and inhibit normal septa formation²¹. Growth of *M. smegmatis* FtsZ-72 in the presence of 0.2% acetamide for 6 h (Fig. 2A, panels iii and iv) led to increased cell length and diminished CL staining at midcell sites and poles as compared with the uninduced cells (Fig. 2A, panels iii and iv with i and ii). Following 18 hrs of induction (Fig. 2A, panels v and vi) cell length was further increased and CL staining was nearly absent; interestingly, most cells also showed defects in cell shape. CL staining was not restored when acetamide was removed and cells were grown in acetamide-free medium for 8 hrs (Fig. 2A, panels vii and viii). Depletion of FtsZ also led to cell elongation and a reduction in CL staining at midcell and poles relative to undepleted cells (Fig. 2B, compare panels iii and iv with i and ii). After 18 hrs (Fig. 2B, panels v and vi) there was an apparent lack of CL staining and cells showed extensive elongation and presence of branches and bud-like structures. Addition of acetamide to these cultures did not recover the CL staining even after 8 hrs growth (Fig. 2B, panels vii and viii). Together these results support a notion

that conditions that interfere with the formation of septa would interfere with CL localization or enrichment.

3.2. Septal staining in *Mtb* synchronous cultures

In the above experiments CL-enriched regions were visualized when cell division was blocked by either the overproduction or lack of FtsZ protein. Next we visualized CL-enriched regions in synchronously replicating *Mtb dnaAcos115* cells. The *dnaAcos115* strain is defective for initiating new rounds of DNA replication at 30°C, a non-permissive temperature, but is proficient at initiating new rounds of active replication and cell division upon shift to 37°C, a permissive temperature²². *Mtb* wild-type cells continue to replicate at both temperatures. Accordingly, wild-type and *dnaAcos115* cells that were incubated at the non-permissive temperature, and at various intervals following a shift to permissive temperature, were stained with NAO. As can be seen, in both cases we observed staining at midcell sites, poles and also at quarter cell positions (Fig. 3A), although the proportions of these structures varied (Fig. 3B).

Actively growing wild-type cells at 37°C showed more midcell staining as compared to *dnaAcos115* (Fig. 3B). After 30 hrs at 30°C, we found similar proportion of staining at midcell sites and poles in *dnaAcos115* whereas relatively higher proportion of midcell staining relative to quarter position in wild-type. The observed staining pattern of wild-type cells is consistent with a notion that these cells are dividing. NAO staining was found to be diminished at 36 hrs post-shift of wild-type cells, presumably due to a decrease in growth rate of the culture as cells enter stationary phase. Unlike the wild-type cells, we found a sharp increase in midcell staining at 12 hrs and a further burst in midcell and quarter position staining at 24 hrs followed by a decrease at 36 h in *dnaAcos115* (see Fig. 3B). A significant proportion of *dnaAcos115* cells showed an increase in cell length at 12 and 24 hrs relative to wild-type cells (see Fig. 3A, compare wild-type images with *dnaAcos115*). The observed rapid burst of staining at midcell and quarter position of *dnaAcos115* supports a notion that the *dnaAcos115* cells are aligned and are synchronized for replication and possibly cell division.

3.3. Localization of the *Mtb* DnaA protein

As reviewed, membrane phospholipids, including CL, promote dissociation of bound nucleotides from DnaA^{6, 12, 26, 27}. These results implied but did not prove that DnaA protein associates with phospholipids. Results presented in the earlier section and elsewhere based on NAO staining revealed that CL-enriched regions are confined to midcell sites and poles¹⁸. Thus DnaA association with CL implies that DnaA localization and CL-enriched regions overlap. To evaluate this possibility, we created a *M. smegmatis* strain expressing *Pami::gfp-dnaA* and visualized DnaA structures by fluorescent microscopy. As can be seen, GFP-DnaA was localized as distinct foci at midcell, quarter cell, and cell poles (Fig. 4A). Note this localization pattern overlaps with CL (Fig. 1). Cell length measurements revealed that 3–4 µm long cells had midcell localizations, whereas 1–2 µm and 5–6 µm cells had mostly polar localizations (Fig. 4B); the calculated Chi square value of 6.77 for these data indicates that a relationship between cell length and DnaA localization is probable. Next we wished to visualize DnaA fluorescent protein in cells stained with NAO. However, because of the technical difficulties associated with the expression and visualization of DnaA-blue fluorescent protein, we could not colocalize DnaA and CL in the same cell. Nonetheless, the similar localization patterns of DnaA and CL tend to suggest that they interact and possibly colocalize.

3.4. Expression of GFP-DnaA as the sole source of DnaA

Next, we asked whether GFP-DnaA could serve as the sole source of DnaA. If GFP-DnaA fusion is functional, then it is likely that the foci we observed are its native localizations and are indeed related to its functional activity within the cell. With this in mind, we swapped the *Pami::dnaA* from a *M. smegmatis dnaA* conditional expression strain with *Pami::gfp-dnaA*; thus providing the fusion protein as the sole source of DnaA in these cells. Analysis of viability data revealed a modest reduction in viability (Fig. 5, $p=0.0138$ compared with wild-type; for merodiploid $p=0.987$) indicating that the *M. smegmatis* GFP-DnaA is functional and can serve as the sole-source for DnaA. However, we did not observe any distinct GFP:DnaA localization in this strain (data not shown).

4. Conclusions

We showed that CL-enriched regions are located at midcell sites and poles of actively dividing cells; that CL-enriched regions could not be detected in cells defective for formation of septa owing to overproduction or depletion of FtsZ. CL is enriched at cell septa during cell division^{28, 29} and it has been shown that CL clustering is promoted by increased cell wall curvature that results from nascent septal synthesis³⁰. Modulation of FtsZ levels has been shown to result in cell division arrest in mycobacteria²¹. Presumably, CL clustering failed to occur in the absence of septa formation under altered FtsZ levels and is likely responsible for the lack of NAO staining (Fig. 2).

Our studies with *Mtb dnaAcos115* cells revealed that NAO could be used to mark septation and cell cycle progression events. For example, we found that, while the wild-type *Mtb* strain showed a random distribution of staining patterns at all time points, the *dnaAcos115* strain showed a significant increase in staining of both midcell and quarter-cell positions at 12 and 24 hrs following return from nonpermissive to the permissive temperature. We can deduce from these results that NAO can be used as an indicator of cell cycle events in synchronous cultures. We also found that DnaA protein localized as distinct foci at midcell, cell poles and possibly at quarter cell positions, much like that seen in *B. subtilis*³¹ and *E. coli*³². Quarter-cell staining corresponds to presumptive daughter cell positions and it is known that under certain growth conditions FtsZ-structures are visualized at these positions^{19–21}. Thus, DNA and CL localization at quarter-cell positions imply that they associate with these regions as soon as the nascent septal synthesis is initiated. It should be noted that one published report indicates that DnaA forms filaments, unlike the localization pattern seen here³³. The localization patterns of DnaA and CL indicate that they interact and possibly colocalize. NAO staining of synchronous populations of cells could give insights into the location of CL-enriched phospholipid domains as a function of cell cycle progression, and in turn, help to shed light on the coordinated positioning of DnaA. DnaA and CL were observed to localize at similar regions of the cell membrane, although further studies are needed to show colocalization and provide some insight as to when their interaction might occur. Together these developments may lead to a greater understanding of cell cycle events, especially those events surrounding the ability of *Mtb* to enter the latent state and subsequently reactivate.

Acknowledgments

This work was supported by NIH grants AI84734 and AI73966 (MM) and AI48417 (MR).

References

1. Tischler AD, McKinney JD. Contrasting persistence strategies in Salmonella and Mycobacterium. *Curr Opin Microbiol.* 2010; 13:93–9. [PubMed: 20056478]

2. Wayne LG, Hayes LG. An in vitro model for sequential study of shift down of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun*. 1996; 64:2062–9. [PubMed: 8675308]
3. Leonard AC, Grimwade JE. Building a bacterial orisome: emergence of new regulatory features for replication origin unwinding. *Mol Microbiol*. 2005; 55:978–85. [PubMed: 15686547]
4. Grimwade JE, Ryan VT, Leonard AC. IHF redistributes bound initiator protein, DnaA, on supercoiled oriC of *Escherichia coli*. *Mol Microbiol*. 2000; 35:835–44. [PubMed: 10692160]
5. Bramhill D, Kornberg A. A model for initiation at origins of DNA replication. *Cell*. 1988; 54:915–8. [PubMed: 2843291]
6. Crooke E. *Escherichia coli* DnaA protein--phospholipid interactions: in vitro and in vivo. *Biochimie*. 2001; 83:19–23. [PubMed: 11254970]
7. Sekimizu K, Bramhill D, Kornberg A. ATP activates dnaA protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. *Cell*. 1987; 50:259–65. [PubMed: 3036372]
8. Mott ML, Berger JM. DNA replication initiation: mechanisms and regulation in bacteria. *Nat Rev Microbiol*. 2007; 5:343–54. [PubMed: 17435790]
9. Kalman LV, Gunsalus RP. Nitrate- and molybdenum-independent signal transduction mutations in narX that alter regulation of anaerobic respiratory genes in *Escherichia coli*. *J Bacteriol*. 1990; 172:7049–56. [PubMed: 2254274]
10. Erzberger JP, Pirruccello MM, Berger JM. The structure of bacterial DnaA: implications for general mechanisms underlying DNA replication initiation. *Embo J*. 2002; 21:4763–73. [PubMed: 12234917]
11. Zawilak A, Kois A, Konopa G, Smulczyk-Krawczynszyn A, Zakrzewska-Czerwinska J. *Mycobacterium tuberculosis* DnaA initiator protein: purification and DNA-binding requirements. *Biochem J*. 2004; 382:247–52. [PubMed: 15137907]
12. Yamamoto K, Muniruzzaman S, Rajagopalan M, Madiraju MV. Modulation of *Mycobacterium tuberculosis* DnaA protein-adenine- nucleotide interactions by acidic phospholipids. *Biochem J*. 2002; 363:305–11. [PubMed: 11931658]
13. Yamamoto K, Moomey M, Rajagopalan M, Madiraju MV. Facilitation of dissociation reaction of nucleotides bound to *Mycobacterium tuberculosis* DnaA. *J Biochem*. 2008; 143:759–64. [PubMed: 18296714]
14. Qin MH, Madiraju MV, Rajagopalan M. Characterization of the functional replication origin of *Mycobacterium tuberculosis*. *Gene*. 1999; 233:121–30. [PubMed: 10375628]
15. Madiraju MV, Moomey M, Neuenschwander PF, Muniruzzaman S, Yamamoto K, Grimwade JE, Rajagopalan M. The intrinsic ATPase activity of *Mycobacterium tuberculosis* DnaA promotes rapid oligomerization of DnaA on oriC. *Mol Microbiol*. 2006; 59:1876–90. [PubMed: 16553890]
16. Dziadek J, Rajagopalan M, Parish T, Kurepina N, Greendyke R, Kreiswirth BN, Madiraju MV. Mutations in the CCGTTCACA DnaA Box of *Mycobacterium tuberculosis* oriC That Abolish Replication of oriC Plasmids Are Tolerated on the Chromosome. *J Bacteriol*. 2002; 184:3848–55. [PubMed: 12081955]
17. Kumar S, Farhana A, Hasnain SE. In-vitro helix opening of *M. tuberculosis* oriC by DnaA occurs at precise location and is inhibited by IciA like protein. *PLoS One*. 2009; 4:e4139. [PubMed: 19127296]
18. Maloney E, Lun S, Stankowska D, Guo H, Rajagopalan M, Bishai WR, Madiraju MV. Alterations in phospholipid catabolism in *Mycobacterium tuberculosis* lysX mutant. *Front Microbiol*. 2011; 2:19. [PubMed: 21552395]
19. Chauhan A, Lofton H, Maloney E, Moore J, Fol M, Madiraju MV, Rajagopalan M. Interference of *Mycobacterium tuberculosis* cell division by Rv2719c, a cell wall hydrolase. *Molecular microbiology*. 2006; 62:132–47. [PubMed: 16942606]
20. Dziadek J, Madiraju MV, Rutherford SA, Atkinson MA, Rajagopalan M. Physiological consequences associated with overproduction of *Mycobacterium tuberculosis* FtsZ in mycobacterial hosts. *Microbiology*. 2002; 148:961–71. [PubMed: 11932443]
21. Dziadek J, Rutherford SA, Madiraju MV, Atkinson MA, Rajagopalan M. Conditional expression of *Mycobacterium smegmatis* ftsZ, an essential cell division gene. *Microbiology*. 2003; 149:1593–603. [PubMed: 12777499]

22. Nair N, Dziedzic R, Greendyke R, Muniruzzaman S, Rajagopalan M, Madiraju MV. Synchronous replication initiation in novel *Mycobacterium tuberculosis* dnaA cold-sensitive mutants. *Mol Microbiol.* 2009; 71:291–304. [PubMed: 19019143]
23. Dziedzic R, Kiran M, Plocinski P, Ziolkiewicz M, Brzostek A, Moomey M, Vadrevu IS, Dziadek J, Madiraju M, Rajagopalan M. *Mycobacterium tuberculosis* ClpX interacts with FtsZ and interferes with FtsZ assembly. *PLoS One.* 2010; 5:e11058. [PubMed: 20625433]
24. Greendyke R, Rajagopalan M, Parish T, Madiraju MV. Conditional expression of *Mycobacterium smegmatis* dnaA, an essential DNA replication gene. *Microbiology.* 2002; 148:3887–900. [PubMed: 12480893]
25. Mingorance J, Rivas G, Velez M, Gomez-Puertas P, Vicente M. Strong FtsZ is with the force: mechanisms to constrict bacteria. *Trends Microbiol.* 2010; 18:348–56. [PubMed: 20598544]
26. Yung BY, Kornberg A. Membrane attachment activates dnaA protein, the initiation protein of chromosome replication in *Escherichia coli*. *Proc Natl Acad Sci U S A.* 1988; 85:7202–5. [PubMed: 2845401]
27. Sekimizu K, Kornberg A. Cardiolipin activation of dnaA protein, the initiation protein of replication in *Escherichia coli*. *J Biol Chem.* 1988; 263:7131–5. [PubMed: 2835364]
28. Mileykovskaya E, Dowhan W. Visualization of phospholipid domains in *Escherichia coli* by using the cardiolipin-specific fluorescent dye 10-N-nonyl acridine orange. *J Bacteriol.* 2000; 182:1172–5. [PubMed: 10648548]
29. Kawai F, Shoda M, Harashima R, Sadaie Y, Hara H, Matsumoto K. Cardiolipin domains in *Bacillus subtilis* marburg membranes. *J Bacteriol.* 2004; 186:1475–83. [PubMed: 14973018]
30. Huang KC, Mukhopadhyay R, Wingreen NS. A curvature-mediated mechanism for localization of lipids to bacterial poles. *PLoS Comput Biol.* 2006; 2:e151. [PubMed: 17096591]
31. Soufo CD, Soufo HJ, Noirot-Gros MF, Steindorf A, Noirot P, Graumann PL. Cell-cycle-dependent spatial sequestration of the DnaA replication initiator protein in *Bacillus subtilis*. *Dev Cell.* 2008; 15:935–41. [PubMed: 19081080]
32. Nozaki S, Niki H, Ogawa T. Replication initiator DnaA of *Escherichia coli* changes its assembly form on the replication origin during the cell cycle. *Journal of bacteriology.* 2009; 191:4807–14. [PubMed: 19502409]
33. Boeneman K, Fossum S, Yang Y, Fingland N, Skarstad K, Crooke E. *Escherichia coli* DnaA forms helical structures along the longitudinal cell axis distinct from MreB filaments. *Molecular microbiology.* 2009; 72:645–57. [PubMed: 19400775]

Fig. 1.

NAO staining of *M. smegmatis* cells. *M. smegmatis* cells were grown in liquid culture from a starting optical density (600 nm) of 0.1 and 200 nM NAO was added 2 hrs prior to observation by microscopy. Exponential phase cultures (top panels) were harvested after 3 hrs and stationary phase cultures (bottom panels) after 20 hrs of growth. The NAO stain binds monoacidic phospholipids in monomer form, but forms a dimer when bound to cardiolipin, which causes a fluorescence shift from green to red. Grey panels – brightfield images, green images - NAO staining of monoacidic phospholipids and red images - cardiolipin staining. Bar = 3 μ m.

Fig. 2.

NAO staining of the *M. smegmatis ftsZ* strains. Actively growing cultures of *M. smegmatis* FtsZ-72 (A) or *M. smegmatis ΔftsZ*, FZ3-78 (B) were grown for 6 hrs (active) or 18 hrs (stationary) with acetamide to induce FtsZ overproduction (A) or without acetamide to induce FtsZ depletion (B). NAO (200 nM) was added in the last 2 hrs of incubation and live cells observed by fluorescence microscopy. Following growth, the culture media was removed by centrifugation and cells suspended in fresh media either without acetamide to reduce overproduction or with acetamide to induce FtsZ production in the depleted strain. Incubation was continued again for 8 hrs with NAO being added for the last 2 hrs. Bar = 3 μm; BF are brightfield images and fluorescence signals in NAO images show regions where cardiolipin is present. Panels i, iii, v and vii of A and B are brightfield images whereas ii, iv, vi and viii are the corresponding fluorescent images.

Fig. 3.

(A) NAO staining of *Mtb dnaAcos115* strain. The NAO dye was added to cultures during a temperature shift experiment and cells were fixed in paraformaldehyde following incubation at either 30°C (non-permissive for growth) or 37°C (permissive temperature). Cells were then observed by fluorescence microscopy. Bar = 3 μm. Fluorescence images (CL) show NAO staining of regions where CL is present. (B) NAO staining patterns observed in *Mtb* wild-type and the *Mtb dnaAcos115* strain. Fluorescence images of NAO stained cells were analyzed and type of staining was recorded for about 100 cells from each strain. Data were plotted in excel.

Fig. 4. GFP-DnaA fluorescent fusion protein localization in *M. smegmatis*. Actively growing *M. smegmatis* Pami::gfp-dnaA cells were induced with acetamide for 1 hr and examined by fluorescent microscopy (**A**). Top panels are brightfield images and bottom panels are fluorescent images showing localization of GFP-DnaA. (**B**) Approximately 100 cells in brightfield images were measured for length and the corresponding fluorescence images were scored for the type of GFP-DnaA localizations.

Fig. 5. Viability of *M. smegmatis* *gfp-dnaA* strains. Cultures were grown to an optical density (OD600) of 0.4, then serially diluted and spread on agar plates. Colonies were counted after 3 days of growth and viability was calculated as the number of CFU per mL of 0.1 OD600