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Replacement of *Mycobacterium smegmatis dnaA* gene by *M. tuberculosis* homolog results in temperature sensitivity

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SUMMARY

The genetic aspects of DnaA mediated initiation of *oriC* replication in mycobacteria are largely unknown. To get insights into the replication initiation process in mycobacteria, we characterized *Mycobacterium tuberculosis* DnaA and its interactions with *oriC*. We show that the replacement of *M. smegmatis dnaA* with the *M. tuberculosis* counterpart expressed from its native promoter resulted in temperature-sensitive (TS) phenotype. However, the TS phenotype was abolished when the *M. tuberculosis dnaA* was expressed from the inducible amidase promoter, which produces elevated levels of DnaA. We provide evidence that *M. tuberculosis dnaA* promoter activity was unaffected at nonpermissive temperature, but the DnaA protein was found to be unstable indicating that protein factors stabilizing *M. tuberculosis* DnaA are absent in *M. smegmatis*. Finally, we show by surface plasmon resonance that the *M. tuberculosis* DnaA interacts with *M. smegmatis oriC*, similar to its cognate *oriC* indicating that the binding interactions between in vitro folded DnaA and *oriC* are unaffected. Our results suggest that *Mtb* DnaA functions as a partially active protein in *M. smegmatis*, hence is not as proficient as *M. smegmatis* counterpart in optimally driving the *M. smegmatis oriC* replication machinery.

1. Introduction

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis, is responsible annually for nearly two million deaths worldwide. *Mtb* is a slow grower with an average doubling time of 24 h and it is generally believed that *Mtb* modulates its growth rates in response to infection. Emergence of multiple-drug resistant and extremely drug resistant strains of *Mtb* globally necessitate an urgent need for the development of new drugs targeted to hitherto unexplored metabolic pathways¹. The genus *Mycobacterium* includes rapid growers such as *M. smegmatis* with average doubling time of 3 hours. The genetic elements responsible for growth rate differences between the two species are unknown. Despite the growth rate differences between the species, *M. smegmatis* serves as an excellent surrogate for evaluating *Mtb* gene functions.

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DNA replication and cell division are essential aspects of cell cycle and both events are critical for cell duplication and multiplication. DNA replication is believed to be regulated at the level of initiation [reviewed in ²⁻⁴]. DnaA protein is the initiator of replication and binds to origin of replication, also known as *oriC*, to initiate replication. *OriC* contains several repeats of nine-nucleotide long DnaA protein recognition sequences, called DnaA-boxes. DnaA protein recognizes and binds to these DnaA-boxes independent of any nucleotide cofactor, but DnaA binding to ATP aids its interactions with DnaA-boxes ²⁻⁵. These initial events lead to rapid oligomerization of DnaA on *oriC* and organization of *oriC*-DnaA initiation complex that results in strand melting, unwinding and DNA synthesis. It has been shown that the *oriC* sequences from closely related bacterial species, viz. members of enterobacteriaceae, can be exchanged indicating a functional similarity within the same family members ⁶⁻⁹. Earlier studies related to mycobacterial replication initiation revealed that mycobacterial *oriC* sequences are essential and show species specificity, i.e. *Mtb oriC* cannot function in *M. smegmatis* and vice versa ^{8, 10}. DnaA proteins of *M. smegmatis* and *M. tuberculosis* share significant sequence identity. Other data indicated that ATP-hydrolysis activity of DnaA protein is critical for DnaA oligomerization on *oriC* and viability of *M. tuberculosis* ¹¹. The *dnaA* promoter (*dnaAp*) region contains several DnaA-boxes ^{8, 10, 12} and is the target of MtrA response regulator ¹³.

The present study was undertaken to gain insights into the DnaA mediated *oriC* replication initiation process of mycobacteria. The following questions were asked: Can *Mtb dnaA* serve as the sole source of DnaA protein in *M. smegmatis* and vice versa? Does *Mtb* DnaA protein interact with *M. smegmatis oriC*? We show that although *Mtb* DnaA binds to *M. smegmatis oriC* in vitro, the replacement of *M. smegmatis dnaA* gene with *Mtb* counterpart results in temperature sensitive (TS) phenotype. Our results favor a hypothesis that the native *Mtb* DnaA protein functions like a partially active protein in *M. smegmatis*.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Escherichia coli Top10 strain was used for cloning purposes and was propagated in Luria Bertani (LB) broth or agar. Recombinants were selected on LB agar containing appropriate antibiotics, viz. ampicillin (Amp) at 100 µg/ ml, kanamycin (Km) at 50 µg/ ml and hygromycin (Hyg) at 100 µg/ ml. *M. smegmatis* and *M. tuberculosis* strains were propagated in Middlebrook 7H9 broth or 7H10 agar supplemented with oleic acid, albumin, dextrose and catalase with appropriate antibiotics as needed (Hyg, 100 µg/ ml and Km, 15 µg/ ml). *M. smegmatis* strains RGM15 and RGM32 have been described earlier ¹⁴. RGM15 is *M. smegmatis dnaA* single crossover (SCO) strain carrying *dnaA* suicidal plasmid pMG25 whereas RGM32 is *M. smegmatis dnaA* mutant strain carrying functional *dnaA* gene expressed from its native promoter integrated at the *attB* locus as described earlier ¹⁴. Growth was monitored by measuring absorbance at 600 nm and viability determined by plating for colony forming units on appropriate media agar plates.

2.2. Homologous recombination and *dnaA* gene replacement

Two-step homologous recombination protocols essentially as described for the construction of *M. smegmatis* and *Mtb dnaA* mutant strains were followed ¹⁴. Briefly, pMG68 plasmid expressing *Mtb dnaA* from its native promoter ¹¹ was integrated into RGM15 strain and screened for double crossovers (DCO) as described at indicated temperatures. In some experiments, pMBL45 plasmid expressing *Mtb dnaA* gene from amidase promoter (*amip*) ¹⁵ was integrated into RGM15 and processed for DCO at 37°C. Characterization of DCOs by PCR and Southern was as described earlier ^{11, 14}.

2.3. Western blotting

M. smegmatis strains grown to exponential phase were spun briefly at 3000X g at 4°C, washed with 10 mM Tris-HCl pH 7.5 and 1 mM EDTA and cell free lysates were prepared by bead-beating as described ¹⁶. Total protein content was determined by BCA protein assay (Pierce) using bovine serum albumin standards. Equal amounts of various protein samples were separated by SDS-PAGE and proteins transferred to nitrocellulose membrane blots. DnaA protein levels in the blots were determined by immunoblotting with purified α -DnaA antibodies utilizing the ECF kit (GE Life sciences) followed by visualization of bands with a Molecular Imager using QuantityOne software.

2.4. Luciferase assay

Luciferase activity was measured as described earlier ¹⁷. The promoter-less *lux* reporter plasmid, pMH66, was generous gift from Dr. David Sherman ¹⁸. To create *dnaAp::lux* construct the *Mtb dnaAp* region spanning the *rpmH-dnaA* intergenic region was PCR amplified using primers MVM458 (5'-TGGCAACACTAGTATCCGGCCACTCACT-3') and MVM459 (5'-GTCGGAAGCTTGTCTCGTTAGTTCCGG-3'). Similarly, to create *sigAp::lux* construct, the *Mtb sigAp* region was amplified using primers MVM480 (5'-CTACTAGACTAGTGCCGGAATTGT-3') and MVM481 (5'-CCACCCAAGCTTTTACTCACTTCCGGT-3'). The PCR products were cloned as *SpeI-HindIII* fragments in pMH66 upstream of *lux* coding region. The reporter plasmids were transformed into RGM32 and RGM71 strains and luciferase activity at indicated time periods was measured by mixing 100 μ l of culture with 100 μ l of 1 mM Beetle luciferin (Promega Corporation) and measured in luminometer (Turner Designs). Luciferase activity was reported as Lux units per unit OD₆₀₀ nm.

2.5. DNA synthesis experiments

DNA synthesis was monitored as described using [5,6-³H] uracil ¹⁴. Briefly, actively growing cultures of RGM32 and RGM71 at 28°C were diluted to low cell density and shifted to 40°C for 6 h. Parallel cultures were grown at 28°C. At the end of 6 h incubation, radiolabeled uracil was added at a final concentration of 0.5 μ Ci/ml, samples were removed at indicated time periods, incubated in 0.3 M KOH for 24 h at 37°C and the amount of radioactivity present was measured.

2.6. Surface plasmon resonance (SPR) techniques

SPR experiments were carried out essentially as described earlier ¹¹. Briefly, biotinylated *oriC* regions of *Mtb* and *M. smegmatis* were generated by PCR and immobilized on SA (streptavidine coated) sensor chip to 60 to 80 resonance units (RU). The *dnaA-dnaN* intergenic region was considered as *oriC* for each species. As a control, scrambled DNA sequence 5'-AAGTAAGTATATAAGTAAGT-3' was coupled and all binding values were subtracted from *oriC* values. Typical binding experiments were carried in 50 mM Tris-acetate pH 8.25, 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM beta-mercaptoethanol, 10 mM ammonium acetate, 0.005% Tween-20, 50 ng poly(dA-dT) per ml, 50 ng sheared salmon sperm DNA/ml and 1 mM indicated nucleotides ATP, ADP or ATP γ S. DnaA was incubated in the above buffer for 5 min and injected over the sensor surface pre-equilibrated in the same buffer. Typical flow rate of samples was 25 μ l per min. Binding association and dissociation were measured as described ¹¹.

2.7. Electrophoretic mobility-shift assay (EMSA)

Samples were prepared on ice in 40 μ l of the above buffer containing radiolabelled *M. tuberculosis oriC* DNA fragment (0.034 μ M) and different concentrations of DnaA protein. We prepared three probes each with a different DnaA-box sequence. Probe 1 sequence

included 5'-CGGCGGTTT**CGTTTACA**ACCCACGC-3' and its complement 5'-GCGTGGGTTGTG-AACGGGAACCGCCG-3'; probe 2 sequence included 5'-GAGTGTCG-**CTGTGCACA**AACCGCGC-3' and its complement sequence 5'-GCGCGGTTTGTGCACAGCGACACTC-3', and probe 3 included 5'-CCCACGCC**TCATCCCCA**CCGACCCA-3' and its complement 5'-TGGGTCGGTGGG-GATGAGGCGTGGG-3'. The presumptive DnaA-box sequences were underlined and shown in boldface. ATP was used at a final concentration of 1 mM. All reactions were carried out in the presence of a 250-fold excess salmon sperm testis DNA. Reactions were incubated at 37 °C for 15 min, mixed with gel-loading buffer containing 50% glycerol and 0.02% bromophenol blue, resolved by PAGE in 5% acrylamide gels and visualized by autoradiography.

3. Results and Discussion

3.1. Replacement of *M. smegmatis* dnaA gene by *Mtb* counterpart results in temperature sensitivity

The primary amino acid sequences of *Mtb* and *M. smegmatis* DnaA show 55 % identity and 59% similarity whereas the nucleotide sequences of the *dnaA*p and the *oriC* show 46% and 49%, similarity, respectively [see also, 8, 10, 12, 19]. In an effort to understand why the *oriC* sequences from these two species cannot be exchanged, we first examined by homologous recombination if the *Mtb* *dnaA* gene can replace *M. smegmatis* counterpart and vice versa. The *dnaA* gene is essential for survival¹⁴, hence its disruption at the native locus requires the presence of a functional copy integrated elsewhere on the chromosome¹⁴. Accordingly, we first integrated pMG68 plasmid expressing the *Mtb* *dnaA* gene from its native promoter at the *attB* locus in *M. smegmatis* *dnaA* SCO strain RGM15. Next, the SCO strain was processed for selection of DCOs at 37°C as described¹⁴. When analyzed by PCR, 80/80 DCOs showed wild-type copy of *dnaA* (Fig. 1A) indicating that *Mtb* *dnaA* cannot replace *M. smegmatis* counterpart under these conditions. We considered a possibility that expression of *Mtb* *dnaA* in *M. smegmatis* could have resulted in toxicity, hence the potential mutant DCOs were nonviable. In such cases, selection of mutant DCOs at conditions that reduce growth rates, i.e. growth at 28°C, avoids issues related to toxicity. Therefore, we selected DCOs at 28°C and the resultant DCOs were replica patched at 42°C. Several DCOs showed TS phenotype and failed to grow at 42°C or 40°C (data not shown). The corresponding colonies growing at 28°C were processed and analyzed by PCR (Fig. 1B) and Southern hybridization (data not shown). All showed a pattern expected for mutant DCO indicating that *Mtb* *dnaA* can replace *M. smegmatis* counterpart provided the growth rate of the DCO was reduced. Presumably, expression of the wild-type *Mtb* *dnaA* in *M. smegmatis* from its native promoter produces a protein that is not proficient at driving *M. smegmatis* *oriC* replication machinery optimally, hence results in loss of viability. One DCO growing at 28°C, designated as RGM71, was characterized further.

3.2. *Mtb* dnaAp activity, but not the DnaA protein levels, was stable at high temperature

To understand the molecular basis for the observed TS phenotype, we evaluated DNA synthesis at nonpermissive temperature (40°C) following 6 h temperature shift and compared results with the cultures growing at permissive temperature (28°C). The 6 h period corresponds to approximately two doubling times for *M. smegmatis*. Parallel experiments were carried out with control strain, RGM32, a DCO expressing *M. smegmatis* *dnaA* at the *attB* locus¹⁴. As can be seen, the DNA synthesis rates of RGM32 growing at 28°C and 40°C were nearly comparable (Fig. 2A). The DNA synthesis at 40°C was linear as opposed to the exponential rate seen at permissive temperature (Fig. 2A). These results suggest that although *Mtb* DnaA functions in *M. smegmatis*, it behaves as a partially active protein, hence is unable to drive *M. smegmatis* *oriC* replication machine optimally at 40°C.

One explanation for the reduced DNA synthesis is that either *Mtb* DnaA protein is unstable at 40°C or that the *Mtb dnaAp* is not optimally active thereby results in reduced DnaA protein levels. Evaluation of DnaA protein levels by immunoblotting revealed a reduction in the DnaA protein levels in the cell lysates of cultures maintained for 1, 2, 4, 8 and 20 h at 40°C as compared to that grown at 28°C (see Fig. 2B) indicating that the protein is not stable at 40°C. In this context, it is unknown if *Mtb* DnaA protein folds properly in *M. smegmatis*.

Next, to evaluate *dnaAp* activity, we created a reporter plasmid expressing *lux* gene downstream of *Mtb dnaAp* (*dnaAp-lux*) and transformed into RGM71 and RGM32 strains. We measured the *dnaAp* activity as relative Lux units at different periods after temperature shift and compared results with the control growing at 28°C (Fig. 2C). Because RGM72 cultures filament and clump at 42°C, Lux activity was measured at 37°C. For control, *lux* gene was also placed downstream of promoter of house keeping gene, *sigA*. As can be seen, the *Mtb dnaAp* activity, expressed as relative Lux units, was same at 28°C and 37°C, like the *sigAp*, in both RGM32 and RGM71 strains (Fig. 2C).

3.3. Elevated production of *Mtb* DnaA protein overcomes TS phenotype

Since DnaA protein levels were reduced upon temp shift, we considered a possibility that the TS phenotype can be reversed if elevated levels of DnaA were present. Accordingly, we expressed *Mtb dnaA* from inducible *amip* and selected for DCOs at 37°C on plates containing acetamide. Earlier studies revealed that expression of *dnaA* gene downstream of *amip* results in 8-fold DnaA accumulation¹⁴. As expected, when *Mtb dnaAp* was expressed from *amip*, we obtained several viable DCOs (Fig. 1C) indicating that the TS phenotype associated with *Mtb dnaA* expression was not an issue in cells producing elevated levels of DnaA.

Together, these results suggest that the *Mtb dnaAp* is well expressed in *M. smegmatis*, but the translated product DnaA is unstable at 40°C. One explanation is that the *Mtb* DnaA protein might not fold properly in *M. smegmatis* and therefore becomes unstable (Fig. 2B). This could lead to a defect in *oriC* replication and bacterial multiplication. Expression from the *amip* results in large amounts of DnaA protein¹⁴ that can counter the inefficient folding and the resulting instability issues at nonpermissive temperatures. It is also possible that hitherto unknown protein cofactors that otherwise promote the stabilization of *Mtb* DnaA protein are either missing or are in insufficient amounts in *M. smegmatis*. Presumably, the regulation of DnaA protein stability in both species could be different, and further studies are required to address this issue.

3.4. Reduction in DnaA protein levels lead to filamentation and cell division blockage

Earlier studies showed that optimal levels of DnaA protein are required for cell cycle progression^{14, 20}. Since DnaA protein levels were reduced following temperature shift, we expected that RGM71 cells would not be proficient for multiplication at 40°C. To evaluate this possibility, we examined the RGM71 cells growing at 28°C, and those following 8h and 12 h shift. The cells were stained with ethidium bromide-mithramycin dyes as described and visualized by brightfield and fluorescence microscopy (Fig. 3). As can be seen, actively dividing cells at 28°C were short (2 to 3 microns in length) and contained two nucleoids per dividing cell (Fig. 3 i and ii). In contrast, RGM71 cells at 40°C were elongated (Fig. 3 iii-vi) and contained multiple nucleoids (Fig. 3 iv and vi). Continued growth at 12 h (Fig. 3 v) and beyond (not shown) led to further increase in cell length and severe clumping. Some of filamentous cells under these growth conditions showed nucleoid-free zones. These results confirm earlier findings that that optimal levels of DnaA protein are required for normal cell cycle progression and reduction in DnaA protein levels lead to cell division blockage^{14, 20}.

3.5. Replacement of *Mtb dnaA* gene by *M. smegmatis* counterpart

In the above experiments we tested whether *Mtb dnaA* gene can replace the function of *M. smegmatis* counterpart. We carried out reciprocal experiments to test whether *M. smegmatis dnaA* can replace *Mtb dnaA* gene function. We transformed pMG25, a *dnaA* suicidal recombination plasmid¹⁴ into *Mtb* expressing *M. smegmatis dnaA* from its own promoter. Screening of DCOs in this background at 37°C showed both wild-type and mutant patterns (data not shown). These results indicate that *M. smegmatis* DnaA is fully functional in *Mtb*. Presumably, the slow growth rate of *Mtb* made the selection of mutant DCOs feasible and this scenario is comparable to permissive temperature growth of *M. smegmatis* (Fig. 1B).

3.6. Interactions of *Mtb* DnaA with *M. smegmatis oriC*

The above data tend to suggest that the *Mtb* DnaA protein can drive *M. smegmatis* DNA replication initiation machinery at 28°C. Using the SPR technique we showed earlier that *Mtb* DnaA protein binds ATP and oligomerizes on *oriC* utilizing its intrinsic ATPase activity¹¹. Furthermore, DnaA-binding to *oriC* in the presence of ATP is biphasic that shows rapid initial 'on' followed by slow association phase, and an initial rapid dissociation 'off' followed by a slow dissociation phase¹¹, whereas the binding in the presence of ADP and ATPγS is monophasic¹¹. Other data indicate that while both *M. smegmatis* and *Mtb oriC* sequences contain multiple DnaA-box repeats, sequences of all DnaA-boxes and their positions are not conserved. Hence, to get insights into the DnaA mediated *oriC* replication initiation process, we evaluated *Mtb* DnaA interactions with *M. smegmatis oriC*.

As a first step, we examined *Mtb* DnaA interactions with different DnaA-boxes by EMSA (Fig. 4A). We showed that *Mtb* DnaA binds and retards the mobility of three different DnaA boxes (Fig. 4A). These results indicate that DnaA protein shows broad sequence specificity, hence could likely interact with *M. smegmatis oriC*. Next, we examined *Mtb* DnaA interactions with *M. smegmatis oriC* by SPR technique in the presence of ATP, ADP or ATPγS. In these experiments, a biotinylated *M. smegmatis oriC* was coupled to streptavidine sensor chip to approximately 80 Response units. In parallel, *Mtb oriC* and a scrambled sequence were also coupled and DnaA interactions with *oriC* were examined. DnaA protein in the presence of indicated nucleotides was then flown over sensor surface and the association and dissociation data were measured. As can be seen, binding isotherms of DnaA with *M. smegmatis oriC* in the presence of different nucleotides were similar to that seen with *Mtb oriC* (Fig. 4B-i). For example, consistent with the earlier published data¹¹, *Mtb* DnaA showed biphasic binding to its *oriC* in the presence of ATP and monophasic binding in the presence of ADP and ATPγS¹¹. Essentially, similar association and dissociation profiles were observed with *M. smegmatis oriC* (Fig. 4B-ii). The biphasic nature of *Mtb* DnaA interaction with *M. smegmatis oriC* is consistent with a notion that *Mtb* DnaA rapidly oligomerizes on *M. smegmatis oriC*, as it does with its cognate *oriC*, presumably utilizing ATP-hydrolysis¹¹. It should be noted the DnaA protein used in these experiments was folded in vitro following denaturation to generate a functionally active protein²¹. Nonetheless, these results indicate that the initial events associated with DnaA-*oriC* interactions between the two species are similar with the in vitro folded protein. It is unknown if these and those steps subsequent to the initial *oriC*-DnaA interaction are modulated in vivo. The differences in the extent of RU between the two species could reflect the different amounts of *oriC* coupled to the sensor chip.

4. Conclusions

Our results reveal that *dnaA* genes in both species can be exchanged, but with some limitations. The TS phenotype associated with the replacement of *M. smegmatis dnaA* function by *Mtb* counterpart suggests that the *Mtb* DnaA behaves like a partially active

protein in *M. smegmatis* and becomes unstable at 37°C and higher. The observed TS phenotype is rather surprising as *Mtb* genes are often used to replace the functions of *M. smegmatis* counterparts and two examples relevant to cell cycle include *ftsZ* and *crgA* ²². Why might wild-type *Mtb* DnaA protein be partially active? One possibility is that the *Mtb* DnaA does not fold well in *M. smegmatis*, thereby making a partially active protein. It is known that DnaA protein interacts with DnaB helicase, another component of the *oriC*-initiation complex. It is also possible that optimal DnaA activity requires its association with hitherto unidentified cofactors, which are missing, or are present in limited amounts in *M. smegmatis*. A consequence would be inefficient activity and reduced stability of the DnaA protein. Optimal levels of DnaA have been shown to be required for regulated cell cycle progression ^{14, 20}. Thus, a reduction in the intracellular levels of DnaA at non-permissive temperature would negatively impact cell cycle progression events. Further studies are required to resolve these issues. I

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Figure 1.

Agar gel analysis of homologous recombination products: (A). Analysis of DCOs obtained at 37°C: *M. smegmatis* SCO strain expressing *Mtb dnaA* from its promoter was processed for selection of DCO at 37°C. Genomic DNA of select DCOs and one SCO were prepared, the *dnaA* gene was PCR amplified, resolved in 0.8 % agarose gels, DNA bands were visualized and photographed. The positions of wild-type marked as 'wt' and mutant marked as 'mut' are shown. The results show all wild-type patterns. (B) Analysis of DCOs obtained at 28°C. All experiments were similar as described above expect that the DCOs were selected at 28°C first and then patched at 42°C. Genomic DNA from cells showing TS was prepared and analyzed as described above. (C). Analysis of DCOs obtained at 37°C. *M. smegmatis* SCO expressing *Mtb dnaA* from *amip* were used to screen for DCOs at 37°C. All other conditions were as described for panel A.

Figure 2.

Characterization of RGM71 DCO. (A) DNA incorporation experiments: RGM71 and RGM32 DCO strains were grown at 28°C and shifted to 40°C was cultured for 6 h. ^3H -Uracil was added and at indicated time periods samples were removed and processed for DNA synthesis as described 14. Y-axis shows the rate of DNA synthesis. (B) Immunoblotting analysis of RGM71 DCOs. RGM71 strain was grown at 28°C and shifted to 40°C. At indicated time periods samples were removed, cellular lysates were prepared, 2 μg protein from each time period were resolved by SDS-PAGE and immunoblotting was performed using α -DnaA antibodies. (C). Luciferase reporter assays: Actively growing RGM71 and RGM32 strains expressing *dnaAp-lux* or *sigAp-lux* at 28°C were shifted to 37°C. At indicated time periods, samples were removed and luciferase activity was measured as described.

Figure 3.

Microscopic analysis of RGM71 cultures: Actively growing cultures of RGM71 at 28°C were shifted to 40°C and continued to grow for 8 and 20 h. Samples were removed, stained with ethidium bromide-mithramycin dyes and visualized by microscopy. Panels i - brightfield and ii - fluorescent images of cultures growing at 28°C; panels iii and v show brightfield images of 8 and 20 h at 40°C whereas iv and vi are the corresponding fluorescent images.

Figure 4.

Mtb DnaA interactions with DnaA boxes (A) and *M. smegmatis* oriC (B).

Panel A: EMSA to visualize DnaA-DnaA box interactions. Radiolabelled DnaA-box fragments were incubated at 37 °C for 15 min with various concentration of DnaA and protein-DNA complexes were resolved by PAGE in 5 % polyacrylamide gels and visualized by autoradiography. A-i is the DnaA-box CCGTTCACA and A-ii – DnaA-box CTGTGCACA and A-iii is DnaA-box TCATCCCCA. Panel (B) SPR analysis of DnaA-oriC interactions. B-i: Biotinylated *Mtb* oriC was coupled to approximately 60 RU on SA sensor chip, and 100 nM DnaA was incubated with 1 mM of indicated nucleotides, flown over the sensor surface and association and dissociations were monitored. B-ii: All experimental conditions were similar to B-i except that *M. smegmatis* oriC was coupled to approximately 80 RU.