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Synchronous replication initiation in novel *Mycobacterium tuberculosis dnaA* cold-sensitive mutants

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Abstract

The genetic aspects of *oriC* replication initiation in *Mycobacterium tuberculosis* are largely unknown. A two-step genetic screen was utilized for isolating *M. tuberculosis dnaA* cold-sensitive (cos) mutants. First, a resident plasmid expressing functional *dnaA* integrated at the *attB* locus in *dnaA* null background was exchanged with an incoming plasmid bearing a mutagenized *dnaA* gene. Next, the mutants that were defective for growth at 30°C, a nonpermissive temperature, but resumed growth and DNA synthesis when shifted to 37°C, a permissive temperature, were subsequently selected. Nucleotide sequencing analysis located mutations to different regions of the *dnaA* gene. Modulation of the growth temperatures led to synchronized DNA synthesis. The *dnaA* expression under synchronized DNA replication conditions continued to increase during the replication period, but decreased thereafter reflecting autoregulation. The *dnaAcos* mutants at 30°C were elongated suggesting that they may possibly be blocked during the cell division. The DnaA115 protein is defective in its ability to interact with ATP at 30°C, but not at 37°C. Our results suggest that the optimal cell cycle progression and replication initiation in *M. tuberculosis* requires that the *dnaA* promoter remains active during the replication period and that the DnaA protein is able to interact with ATP.

Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, is responsible for approximately 1.7 million deaths each year. Tuberculosis infections are also the leading cause of morbidity and mortality in AIDS patients. *M. tuberculosis* is a slow grower with an average doubling time of 24 h. Its efficiency as an infectious agent relies on its ability to shift between two physiologically distinct growth states upon infection including an active replicative state with an increase in the bacterial burden and a latent or quiescent state characterized by a limited bacterial turnover. It is believed that the bacterium in the latter state remains metabolically active, but in a state of non-replicative persistence (NRP) (Wayne and Hayes, 1996). The NRP bacteria reactivate and resume active replication under conditions that are favourable for their growth, and multiply to cause an infection. DNA replication, which is a critical aspect of the cell cycle, is essential for cell duplication. Although the *M. tuberculosis* genome sequence was determined nearly a decade ago (Cole *et al.*, 1998), the genetic aspects of the regulation of DNA replication in this medically important pathogen are largely unknown.

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DNA replication is believed to be initiated when the DnaA protein recognizes and binds to repeats of specific sequences consisting of nine nucleotides, called DnaA boxes, located in the origin of replication (*oriC*) (Bramhill and Kornberg, 1988a, 1988b). Binding of the ATP-bound form of DnaA to these DnaA-boxes triggers a cascade of events that result in the melting of the *oriC* DNA region, the assembly of additional proteins of the *oriC*-DnaA complex, and ultimately the unwinding and synthesis of DNA. Extensive genetic and biochemical studies dealing with *dnaA* and *oriC* have revealed that *E. coli* employs multiple mechanisms to prevent multiple initiations at *oriC* such that the “once-per-cell-cycle initiation at any given time” rule is maintained (Skarstad *et al.*, 1986; Skarstad *et al.*, 1988). These mechanisms include: (i) sequestration of the *dnaA* promoter so that de novo synthesis of DnaA is prevented (Campbell and Kleckner, 1990; Theisen *et al.*, 1993); (ii) sequestration of the DnaA protein away from the *oriC* toward newly replicated chromosomal elements, which provides a sink for DnaA (Kitagawa *et al.*, 1996; Kitagawa *et al.*, 1998; Morigen *et al.*, 2001); (iii) inactivation of DnaA by stimulating its intrinsic ATPase activity by both the DnaN and Hda proteins via a process called regulatory inactivation of DnaA (RIDA) (Katayama *et al.*, 1998; Kurokawa *et al.*, 1999); and finally (iv) sequestration of *oriC* by SeqA, which shows high affinity for hemimethylated DNA and binds GATC sites that are abundantly present in the *oriC* region (Baker, 1994; Lu *et al.*, 1994; Slater *et al.*, 1995). All of these mechanisms lead to either modulation of DnaA activity and or levels of DnaA protein in addition to the reduction of *oriC* availability.

Earlier studies on DnaA mediated *oriC* replication in *M. tuberculosis* have revealed that both DnaA_{TB} and *oriC* are essential and that DnaA_{TB} is a DnaA-box binding protein (Dziadek *et al.*, 2002; Madiraju *et al.*, 2006; Qin *et al.*, 1999; Yamamoto *et al.*, 2002a; Zawilak *et al.*, 2004). The DnaA_{TB} protein exhibits weak ATPase activity (Yamamoto *et al.*, 2002a; Yamamoto *et al.*, 2008) and uses its intrinsic ATPase activity to rapidly oligomerize at *oriC* (Madiraju *et al.*, 2006). The *M. tuberculosis* strain producing DnaA that is defective for ATPase activity, but is proficient for binding to ATP, is inviable (Madiraju *et al.*, 2006). It is unknown how *oriC* replication mediated by DnaA is regulated in *M. tuberculosis* and whether or not *oriC* replication in *M. tuberculosis* involves any or all of the aforementioned regulatory mechanisms. The availability of well-defined and genetically characterized *M. tuberculosis dnaA* mutants will facilitate the dissection of the initiation process and will help us to begin understanding the regulatory mechanisms operating in this organism. To begin addressing this issue, we have developed a simple and robust protocol for isolating several novel alleles of *M. tuberculosis dnaA* strains that demonstrate a cold-sensitive (cos) phenotype. Characterization of these mutants revealed that DNA replication initiation can be synchronized by modulating the growth temperatures and that optimal cell cycle progression requires that *dnaA* expression remain high during the DNA synthesis period.

Results

M. tuberculosis dnaAcos mutants

A two-step genetic screen in a *dnaA* strain background was developed for isolating *M. tuberculosis dnaAcos* mutants. The *dnaA* strain is a chromosomal null for *dnaA* but contains an L5 phage based vector carrying a functional copy of *dnaA* integrated at *attB* locus (*attB::dnaA*) (Madiraju *et al.*, 2006). The *dnaA* at *attB* locus in this strain was located approximately 2.7 Mbp relative to its native location. Thus, the *dnaA* gene in this strain is expected to be replicated with a significant delay relative to *oriC*, since it is close to the end of the replication period.

In the first step, the *dnaA* strain was electrottransformed with a plasmid DNA library expressing mutant *dnaA* genes. Since resident mycobacterial L5-based integrating vectors can be very efficiently excised with an incoming plasmid carrying a different antibiotic

marker (Pashley and Parish, 2003), this step could result in the replacement of the integrated plasmid carrying a functional copy of *dnaA* with that of the incoming plasmid expressing the mutant copy. In the second step, the *dnaA* transformants that were viable at 37°C (permissive temperature), but not at 30°C (nonpermissive temperature), were selected by replica patching and were characterized further. Using this protocol, several viable transformants were obtained and 7 out of the 3300 analyzed were found to be consistently cold sensitive (cos) (Fig. 1A). These cos transformants recovered readily when shifted to the permissive temperature (Fig. 1B). Since the coding region of the *dnaA* gene and not its promoter was PCR mutagenized in our protocol, we would expect that the cos phenotype could be due to an altered activity of the DnaA protein, but not due to altered expression levels of *dnaA*. DnaA protein autoregulates its expression (Braun *et al.*, 1985). Thus, it is possible that mutations in the *dnaA* coding region affect its ability to regulate its own expression and therefore DnaA levels. Evaluation of the *dnaA* transcript and the translated product could provide additional insights into these issues.

Nucleotide sequence analysis identified that the mutations occurred in different regions of the *dnaA* gene (Fig. 1C). With the exception of *dnaA231*, all of the strains contained multiple mutations. The *dnaA231* strain contained a single point mutation at codon 375 and the affected residue is part of a region that is believed to be important for its ATPase activity. Strains *dnaA108*, *dnaA115*, *dnaA132*, *dnaA183* and *dnaA255* carried an identical set of mutations at codons 73, 151 and 215. However, strain *dnaA255* also possessed another mutation at codon 468. Interestingly, codons 215 and 468 are in regions believed to be important for the binding of ATP and DNA, respectively (Erzberger *et al.*, 2002; Roth and Messer, 1995, 1998; Schaper and Messer, 1995). Finally, strain *dnaA245* possessed three mutations, but all of these are at different sites (Fig. 1C).

Characterization of the *dnaAcos* mutants

The *dnaAcos* mutants showed reduced viability (Fig. 1D) and filamentation at the nonpermissive temperature, but recovered when shifted to the permissive temperature (Fig. 1E shows the phenotype of the representative strain *dnaA115*). Measurement of the incorporation of radiolabeled uracil into alkali stable DNA revealed that *dnaAcos* mutants were as proficient as the wildtype strain in synthesizing DNA at 37°C (Fig. 2A), but were defective at 30°C (Fig. 2B). DNA synthesis in *dnaAcos* cultures at 30°C showed a small initial increase and reached a plateau between 16h (*dnaA115*) to 34h (*dnaA231* and *dnaA245*). The observed gradual plateau is possibly due to a heterogeneous population of cells completing ongoing rounds of DNA synthesis that were unable to initiate new rounds of DNA synthesis. In contrast, DNA synthesis in wildtype cells continued to increase at 30°C (Fig. 2B). These data suggest that the *dnaAcos* mutants are defective for reinitiation at 30°C. It should be noted that the wildtype strain in these and other experiments described below refers to the parent strain wherein the *dnaA* gene is located at its native location. Hence the wildtype strain does not constitute an identical control to *dnaAcos* cells that contained a functional copy of the *dnaA* gene at the *attB* site. However, the location of the *dnaA* gene is not expected to affect the outcome of the experiments presented in this study.

DNA synthesis in *dnaAcos* mutants is aligned

The DNA synthesis patterns of *dnaAcos* mutants at the nonpermissive temperature (Fig. 2) combined with the reversal of the cold-sensitive phenotype upon a temperature shift (Fig. 1D and E) suggested a possibility that DNA replication in these mutants can be aligned by modulating the growth temperatures. Accordingly, experiments as shown in Fig. 3A were performed. In these experiments, two identical sets of cultures actively growing at 37°C were transferred to 30°C (marked as I in Fig. 3A) and incubated for 26h prior to shifting the cultures back to 37°C allowing for DNA synthesis (Fig. 3A, marked as 'II'). The 26h

incubation period at 37°C corresponds to approximately one doubling time for the culture. Preliminary experiments established that *dnaAcos* cultures growing at 30°C were able to resume DNA synthesis with a 2h lag period when the cultures were returned to 37°C (not shown). Therefore, 2h after incubation at 37°C, one set of cultures was returned to 30°C (see Fig. 3A, marked as III) and the other remained at 37°C. DNA synthesis was followed at both temperatures.

As can be seen, cultures that were returned to 30°C after the 2h incubation period at 37°C continued DNA synthesis for approximately 11h where it reached a plateau (see Fig. 3B, time point at II is marked as '0'). This portion of the curve is referred to as the DNA synthesis period, "C". An additional burst of DNA synthesis occurred only when a second temperature shift experiment was performed by transferring the culture to 37°C for 2h and then transferring these cultures back to 30°C (see Fig. 3B, marked as IV and V, respectively at 26 and 28 h). Again, DNA synthesis continued for 11h and reached a plateau thereafter. Each burst of DNA synthesis led to a doubling in the amount of radioactivity indicating that bacteria were able to complete one round of DNA synthesis, but failed to initiate new rounds at 30°C. These results suggest that the C period at 30°C is approximately 11h in length. In contrast, the wildtype strain continued to synthesize DNA in a linear fashion without requiring temperature shifts.

The *dnaAcos* cultures maintained at 37°C showed a similar trend with the exception that the new burst of DNA synthesis occurred after roughly 24h (Fig. 3C time point at II marked as '0'). As in the situation observed at 30°C, DNA synthesis continued for approximately 10h, reached a plateau and underwent a burst at about 24h, which again continued for a period of about 10h (Fig. 3C). Here, as observed previously, the radioactivity was doubled in each cycle. The plateau period between the two bursts of DNA synthesis at 37°C may include 'D' in addition to any of the 'B' periods. The latter period corresponds to the time after cell division that precedes the initiation of a new round of DNA replication. Evaluation of the cell number determinations at various periods during the cell cycle could enable us to determine the 'D' and 'B' periods precisely. As can be seen, the wildtype cells continued DNA synthesis that proceeded in a linear manner. Analysis of the DNA synthesis data revealed a rate of ~ 3030 nt/min at 30°C and 3333 nt/min at 37°C (see Fig. 3 legend for details).

In the aforementioned experiments, cultures were incubated for 26h at 30°C prior to the temperature shift. Identical results, with respect to DNA synthesis (see above) and *dnaA* expression (see below), were also obtained when the incubation period of the cultures at 30°C was continued for 30h (data not shown). Incubation periods longer than 30h were not performed since these cultures became filamentous and clumpy. From the above results we may conclude that modulation of the growth temperatures of *dnaAcos* mutants lead to synchronized replication initiation in *M. tuberculosis*.

Rifampicin, an inhibitor of RNA polymerase and DNA transcription, has been shown to inhibit initiation of new rounds of DNA synthesis (Lark, 1972; Messer, 1972). Accordingly, we evaluated if the addition of rifampicin at the time of temperature shift interferes with *oriC* initiation and subsequent DNA synthesis at 37°C. The addition of rifampicin allowed the initial round of DNA synthesis to proceed but prevented a second burst of DNA synthesis (supplementary Figure S1). Since *dnaA* transcription is active even at 30°C (see below), we interpret the data to mean that the necessary DnaA potential sufficient for the initiation of one round of DNA replication may have been accumulated during incubation at 30°C. However, addition of rifampicin at the time of temperature shift prevented new rounds of *dnaA* transcription and hence prevented the second burst of initiation and subsequent DNA synthesis.

***dnaA* expression under synchronized replication initiation conditions**

De novo expression of *dnaA* is believed to be one of the factors that are important for the initiation of new rounds of DNA synthesis. Hence, the kinetics of *dnaA* expression under synchronized replication initiation conditions (see Fig. 3) was examined. RNA samples at various periods after temperature shift, in addition to samples taken prior to the shift, were prepared and *dnaA* expression relative to 16S rRNA was evaluated (Fig. 4). Since the *dnaA* expression profiles at both 30 and 37°C were identical, the results obtained under only one condition are shown. The following observations were evident: (1) *dnaA* expression in the wildtype and *dnaAcos* strains was reduced at 30°C when compared to expression at 37°C. It is not evident why *dnaA* expression levels decreased at 30°C, however one possibility is that the affinity of DnaA to the DnaA boxes at 30°C is higher than at 37°C resulting in tighter repression of *dnaA* promoter. (2) *dnaA* expression levels in the wildtype strain returned to normal levels upon performing a temperature shift that was maintained thereafter, although a small reduction was noted at 16h. The 16h time point corresponds to the post-replication period and since increased levels of *dnaA* are not expected to be required, the *dnaA* expression was repressed.

(3) *dnaA* expression in strains of *dnaAcos* mutants show two distinct features including an elevated expression level during the C period (8h) in addition to a rapid reduction thereafter. It should be noted that the *dnaAcos* gene located at the *attB* locus is within close proximity to the midpoint of the genome [~ 2.7 Mbp of 4.4 Mbp]. Accordingly, one would expect that duplication of *dnaA* gene would occur toward the end of the C period at approximately 9h. Presumably, the increased expression of *dnaA* during the C period is not due to gene duplication, but rather due to an increased requirement of *dnaA*. To address this question, we examined the expression profile of the wildtype *dnaA* gene located at *attB* under identical growth conditions (supplementary Figure S2). The *attB::dnaA* expression profile was similar to that of the strain that has the *dnaA* gene located at its native locus next to *oriC*, again confirming that the increased expression of *dnaA* during the C period in synchronized *dnaAcos* mutants is not due to gene duplication or relocation of the gene, but rather due to a requirement for increased *dnaA* expression (see below and discussion). We propose that the rapid reduction in *dnaA* expression following *dnaA* gene duplication (approximately 9h) is due to the autoregulatory activity of DnaA, the sequestration of its promoter by hitherto unidentified regulatory proteins (see discussion), or some combination of both mechanisms.

Addition of chloramphenicol, a protein synthesis inhibitor, at the time of the temperature shift, did not interfere with *dnaA* expression (supplementary Figure S3). We also examined the expression pattern of the essential *mtrA* response regulator under these conditions. Earlier studies had indicated that the *dnaA* promoter is the MtrA response regulator target (Fol *et al.*, 2006). Interestingly, our results demonstrated that the *mtrA* expression remained relatively similar at all growth periods indicating that similar expression levels of *mtrA* are required at all periods of the cell cycle (Fig. 4B).

Intracellular levels of DnaAcos remain stable

We considered the possibility that the cold-sensitive phenotype of the *dnaAcos* mutants is due to alterations in the levels or possibly in the activity of the DnaA protein. To obtain insights into this issue, we examined the status of DnaA by immunoblotting in *dnaAcos* mutants growing at 30°C and 37°C and compared these results to analogous assays performed with the wildtype strain. SigA was used as an internal control (Fig. 5A, see panels I and ii). Quantitation of the band intensities confirmed that the relative ratios of DnaA:SigA were similar at both temperatures and in all strains (Fig. 5B). Interestingly, the DnaA:SigA ratios of *dnaAcos* strains were lower than that observed of the wild type strain.

Although the reason for this is not evident, nonetheless, these results support a notion that both the cold-sensitive phenotype (Fig. 1) and the inability to initiate new rounds of DNA synthesis (Fig. 3) at the nonpermissive temperature is not due to altered levels of DnaA.

The DnaA115 protein is defective for binding to ATP at 30°C

Initial interactions of DnaA with ATP are critical for the formation of a productive *oriC*-DnaA initiation complex that is competent for replication initiation (Bramhill and Kornberg, 1988a). Hence, we considered the possibility that the *cos* phenotype of the *dnaAcos* mutants is due to an altered DnaA activity. This is not an unreasonable assumption since some of the *dnaAcos* mutants, for example *dnaA108*, *dnaA115*, *dnaA132*, *dnaA183* and *dnaA255*, had mutations in the conserved GxGxxGKT motif that is critical for the binding of ATP (Walker *et al.*, 1982). It is important to note that the *E. coli dnaA46* temperature sensitive (*ts*) mutant, which is reversible in its thermostability, is defective for the binding of ATP (Hwang and Kaguni, 1988a, 1988b). Hence, in order to test this prediction, we overproduced the mutant DnaA as his-fusion proteins and attempted to purify the proteins using nickel affinity columns. Notably, we were able to purify DnaA115 but not DnaA231 and DnaA255 due to observed aggregation problems. The ability of DnaA115 to bind ATP at 30 and 37°C was examined using filter binding assays (Fig. 6). The wildtype DnaA protein bound ATP at both 37 and 30°C with similar affinities ($K_d \sim 146$ nM). In contrast, DnaA115 was proficient for the binding of ATP at 37°C ($K_d \sim 6.6$ μ M), but was completely defective at 30°C (Fig. 6). DnaA115 showed a sigmoidal binding tendency at 37°C. Mutations at other locations in DnaA115 may have affected the overall ability of this mutant protein to interact with ATP.

dnaA115 growing at the nonpermissive temperature is blocked at cell division

The *dnaA* expression patterns of *dnaAcos* and the altered interactions of DnaA115 with ATP at 30°C suggest that *de novo* synthesis of DnaA that is competent to interact with ATP, is necessary for cell cycle progression. We have shown that *dnaAcos* mutants at the nonpermissive temperature were not proficient for the initiation of new rounds of DNA synthesis (Fig. 3) presumably due to defective interactions between DnaA and ATP (Fig. 6). These cells, however, would be expected to be proficient for cell division. Thus, continuous growth at the nonpermissive temperature could lead to filamentous cells with single nucleoids. To test this prediction we visualized the cell morphology by using brightfield microscopy (Fig. 7, panels marked as 'I') and visualized the nucleoids by using fluorescence microscopy following staining with a mixture of ethidium bromide and mithramycinA (Fig. 7, panels marked as 'ii'). The wildtype cells growing at 30°C and 37°C and the *dnaAcos* cells growing at 37°C were nearly similar in size with one to two nucleoids per cell (Fig. 7). In contrast, the *dnaA115* cells growing at 30°C were elongated and blocked at cell division (Fig. 7). The DNA staining pattern indicated that the nucleoids in the *dnaA115* cells at 30°C were diffuse, as though they contained more than two nucleoids per filamentous cell. Certainly, more sensitive methods such as flow-cytometry and microarray techniques are required to determine the total number of origin copies per cell. Continued incubation of *dnaA115* at 30°C increased the cell length although the majority of these cells contained diffuse nucleoids with nucleoid-free zones (see supplementary Figure S4). Together, these results indicate that the optimal cell cycle progression in *M. tuberculosis* requires a DnaA protein that is competent to bind and possibly hydrolyze ATP in order to catalyze other activities.

Discussion

M. tuberculosis dnaAcos versus the *E. coli dnaA* conditional lethal mutants

DnaA, a member of AAA+ family ATPases, is a highly conserved protein in eubacteria (reviewed in (Erzberger *et al.*, 2002)), and is recognized to contain several functional

domains that are implicated in oligomerization, association with DnaB, ATP, polar lipids and DNA (Messer, 2002; Schaper and Messer, 1997). The conditional lethal *M. tuberculosis dnaAcos* mutants that we have isolated possessed mutations distributed in all these regions of the DnaA protein (Fig. 1C) and importantly, the affected residues are conserved in eubacteria (see supplementary Figure S5).

While we obtained a single point mutation in the conserved box VII of the Walker type B ATPase motif, RELEGA (Neuwald *et al.*, 1999), we did not obtain a single point mutation in the ATP-binding and DNA-binding motifs (see Fig. 1C, compare *dnaA231* with *dnaA108*, *dnaA115*, *dnaA132*, *dnaA183* and *dnaA255*). Failure to obtain a single point mutation in the ATP- or DNA-binding regions suggest that mutations in this region could result in a defective protein that renders the cell inviable and that mutations located in other regions may suppress this phenotype. For the sake of comparison, classic *E. coli dnaA* mutants, including *dnaA46* and *dnaA5*, that bear mutations in the ATP-binding pocket carry a secondary mutation elsewhere in the gene (Hansen *et al.*, 1992). An alternate possibility, although unlikely, is that our mutation search is not exhaustive.

The *M. tuberculosis dnaAcos* mutants, unlike the *E. coli dnaA46* (ts), show a 'delayed replication defect' (slow stop) at the non-permissive temperature, but recover readily at the permissive temperature (Abe and Tomizawa, 1971; Evans *et al.*, 1979). DNA synthesis in *dnaA46* strains can be aligned if cells are allowed to accumulate excess initiation potential at 42°C prior to returning to the permissive temperature. *E. coli dnaAcos* is a spontaneous revertant of the *dnaA46* (ts) mutation, and carries two intragenic suppressor mutations (Braun *et al.*, 1987). This mutant shows excessive reinitiations (Kellenberger-Gujer *et al.*, 1978), presumably due to defective interactions with ATP (Simmons and Kaguni, 2003). Our results suggest that the *M. tuberculosis dnaA115* and possibly other *dnaAcos* mutants are comparable to the *E. coli* reversible type of mutants, such as *dnaA46*.

We showed that *dnaA115* carries three mutations (Fig. 1C). However, it is unknown which of these mutations is responsible for the observed phenotypes such as cold sensitivity, defective binding to ATP, and an ability to exhibit synchronous bursts of DNA synthesis at the permissive temperature following incubation at the non-permissive temperature. Evaluation of the contribution of these mutations individually and together in various combinations will provide valuable clues to the contribution of the amino acid residues to the replication initiation process in *M. tuberculosis*. Our future studies will address these important yet unresolved questions.

Synchronized DNA replication and initiation once-per-cell cycle

Results presented in this study reveal (Figs. 3 and 4) several features associated with *oriC* replication in *M. tuberculosis*. First, initiation of DNA replication in *M. tuberculosis*, as in *E. coli*, requires *de novo* DnaA synthesis. This claim is substantiated by our findings that the addition of rifampin enabled the completion of on-going rounds of DNA synthesis, but did not allow for the start of new rounds of DNA synthesis (Fig. S2). Second, although *dnaA* transcription and translation occur at both 37 and 30 °C, only DnaA competent to interact with ATP was found to be proficient for initiation (Figs. 3 and 4). The inability of DnaA115 to bind ATP at 30°C explains why *dnaAcos* is not proficient for the second-round of reinitiation at 30°C (Fig. 3).

Third, optimal cell cycle progression and viability require that the *dnaA* promoter remains active during the entire cell cycle, with elevated activity during the C period and reduced activity immediately thereafter. The *dnaA* gene in the *dnaAcos* mutants is expected to duplicate just prior to the end of the C period. Thus, reduction in the *dnaA* expression levels following *dnaA* gene duplication, therefore at the end of the C period, could either be due to

an autoregulation event or due to the sequestration of the *dnaA* promoter (see below). Since *dnaA* cells at the nonpermissive temperature are filamentous and possibly blocked at cell division (Fig. 7), we predict that elevated *dnaA* expression during the C period provides the necessary pools of active DnaA required for regulated transcription of genes involved in cell division and other processes. This is not an unreasonable assumption since the transcriptional regulatory role for DnaA has been well recognized (Messer and Weigel, 1997). *M. tuberculosis* DnaA, like its *E. coli* counterpart, recognizes and binds to DnaA-boxes (Dziadek *et al.*, 2002; Madiraju *et al.*, 2006). Our bioinformatics analysis identified the DnaA-box like sequences in the putative promoters of genes involved in cell division and other metabolic pathways such as lipid biosynthesis and nucleotide biosynthesis (not shown). Presumably, the interactions of DnaA with the target promoter sequences results in the temporal expression of genes involved in septation and cell cycle progression. These results substantiate our earlier report showing that overproduction or conditional depletion of DnaA in *M. smegmatis* produces filamentous cells blocked at cell division (Greendyke *et al.*, 2002).

Finally, the nucleotide (ATP) bound state of DnaA is important for cell cycle progression and the initiation of DNA synthesis. This is because even though transcription and translation of *M. tuberculosis dnaA115* proceed at 37 and 30°C, new rounds of DNA synthesis were observed only at 37°C. *M. tuberculosis* DnaA binds ATP and ADP with relatively high affinity (Yamamoto *et al.*, 2002a; Yamamoto *et al.*, 2002b), but only DnaA protein competent to bind and hydrolyze ATP promotes the rapid oligomerization required for the initiation of replication at *oriC* (Madiraju *et al.*, 2006). Since DnaA115 protein is defective for binding to ATP at 30°C (Fig. 6), this could explain why the *dnaA115* strain failed to reinitiate DNA synthesis at the non-permissive temperature (Fig. 3).

The observed 10h C period in synchronized population of *M. tuberculosis* is longer than we expected, but is consistent with an earlier report describing the DNA synthesis time in *M. tuberculosis* (Hiriyanna and Ramakrishnan, 1986). The C period in *E. coli* is also long when grown in chemostats and in media containing succinate as opposed to glucose, although the reasons for this are unknown (Bipatnath *et al.*, 1998). The long C period in *M. tuberculosis* with a $t_D=24$ h is not due to the presence of single *rrn* operon in its genome because *M. marinum* with $t_D=3$ h has also one *rrn* operon (Helguera-Repetto *et al.*, 2004). It is possible that DnaA could regulate the intracellular levels of nucleotide pools, hence the overall rate of DNA synthesis. Alternatively, lagging strand DNA synthesis could be slow in *M. tuberculosis*. Clearly, further studies are required to understand this issue. It is pertinent to note that the slow growth rate of *M. tuberculosis* is also correlated to the presence of large number of insertion sequences in its genome (Cox, 2004).

The *M. tuberculosis dnaA* promoter contains several DnaA-box like sequences (Qin *et al.*, 1999). The binding of DnaA to these DnaA boxes immediately after gene duplication could lead to regulated expression of *dnaA*, similar to the reported autoregulatory activity of *E. coli* DnaA (Braun *et al.*, 1985). We have recently shown that the MtrA response regulator promotes *dnaA* transcription by binding to the *dnaA* promoter in a phosphorylation-dependent manner (Fol *et al.*, 2006). Thus, another possibility is that intracellular ratio of the phosphorylated to the non-phosphorylated MtrA response regulators dictates the outcome of *dnaA* expression. A consequence would be the regulation of *dnaA* expression.

DNA synthesis under synchronized replication conditions reveal that hyper-initiation following reinitiation does not occur in *M. tuberculosis* (Fig. 3C). This raises the question as to how 'initiation once-per-cell cycle' is achieved in this organism. Studies with *E. coli* reveal that DnaA mediated *oriC* replication is initiated when a threshold level of DnaA within a cell is reached (Lobner-Olesen *et al.*, 1989; Skarstad *et al.*, 1986; Skarstad *et al.*,

1989), and that a sequestration of both the *dnaA* promoter and the *oriC* regions are required for achieving the controlled once-per-cell-cycle initiation [reviewed in (Riber and Lobner-Olesen, 2005)].

The *M. tuberculosis* *oriC* does not contain GATC methylation sequences and the genome does not encode for the SeqA-like protein. Our earlier data show that the active species for initiating replication at *oriC* is the DnaA protein that is capable of binding and hydrolyzing ATP (Madiraju *et al.*, 2006). Hence, in order to explain how *oriC* initiation in *M. tuberculosis* is regulated, we propose that DnaA-box like sequences present in the promoters of several genes including the *dnaA* gene provide a sink for DnaA-ATP pools remaining after initiation thereby minimizing the active DnaA threshold available for reinitiation. We have recently shown that the intrinsic ATPase activity of DnaA promotes rapid oligomerization of DnaA-ATP complexes on *oriC* (Madiraju *et al.*, 2006). This process may result in the conversion of DnaA-ATP complexes to DnaA-ADP complexes (Madiraju *et al.*, 2006). Thus, another possibility is that a tight association of DnaA-ADP complexes with *oriC* following its rapid oligomerization prevents the reinitiation from *oriC* (Madiraju *et al.*, 2006). This situation may be analogous to that of the RIDA mechanism (Katayama *et al.*, 1998). *M. tuberculosis* DnaA is membrane associated (Yamamoto *et al.*, 2008) and acidic phospholipids promote dissociation of nucleotides from DnaA and possibly restore levels of active DnaA (Yamamoto *et al.*, 2002a; Yamamoto *et al.*, 2008). Finally, in addition to DnaA, other hitherto unidentified regulatory proteins may target both the *dnaA* promoter and the *oriC* in order to regulate hyper-initiation. One such regulator could be MtrA (Fol *et al.*, 2006). Further characterization of *dnaAcos* mutants could provide valuable insights into the regulation of *oriC* replication process in *M. tuberculosis*.

Finally, investigations in DNA replication regulation and cell cycle progression in *M. tuberculosis* remain relatively unexplored as compared to those in other organisms. Larry Wayne developed a hypoxia-based culture system where actively growing *M. tuberculosis* are subject to a gradual hypoxic state over a period of two to three weeks in sealed, air-tight tubes in order to convert the population to a non-replicative persistent state. Hypoxic *M. tuberculosis* cells are shown to catalyze synchronous replication upon resuspension of cultures in fresh oxygenated media (Wayne, 1977; Wayne and Hayes, 1996). These are time consuming experiments, and the status of the *dnaA* promoter activity following reactivation and cell cycle progression have not been investigated. The simple growth temperature shifts for aligning DNA synthesis with the *dnaA* mutants that we generated should prove useful in investigating the regulatory mechanisms involved in *M. tuberculosis* DNA replication and cell cycle.

Materials and Methods

Bacterial strains and culture conditions

The *E. coli* strain Top-10 (Stratagene) and *M. tuberculosis* strain H37Ra was used in all of the experiments presented herein. Middlebrook 7H9 broth supplemented with oleic acid, albumin, dextrose and sodium chloride was used to culture *M. tuberculosis*. Transformants were selected on media supplemented with agar. In order to select for transformants, hygromycin was used at either 50 $\mu\text{g ml}^{-1}$ for *M. tuberculosis* or 100 $\mu\text{g ml}^{-1}$ for *E. coli*. In addition, kanamycin was used at either 10 $\mu\text{g ml}^{-1}$ for *M. tuberculosis* or 50 $\mu\text{g/ml}$ for *E. coli* to select transformants.

Protocol for creating the *dnaAcos* mutants

Our protocol for constructing the *M. tuberculosis* *dnaA* mutants involved several steps. In the first step, we created the PCR mutagenized plasmid *dnaA* library wherein the mutated

dnaA coding region was cloned downstream of its native promoter. The native *dnaA* promoter was amplified using primers 5'-CCATCGATCGCCGGTTGTTTCGGCTGGAAGGTC-3' (MVM372) and 5'-TTGCAAGCTTCATATGCGTATCTCCCTGGTTCTC-3' (MVM408), with *Cla*I, *Hind*III, and *Nde*I sites, respectively, underlined. The PCR fragments that were amplified were cloned into a kanamycin-resistant pMV306 vector digested with the same enzymes. Second, the *dnaA* coding region was amplified using primers 5'-CGCCTGCAGCATATGGCCGATGACCCCG GTTC-3' (Q58) and 5'-TGCTCTAGACTACTAGCGCTTGGAGCGCTGAC-3' (MVM379) with *Nde*I and *Xba*I sites, respectively, in the presence of 1.5 mM MgCl₂ and 0.5 mM MnCl₂, Taq DNA polymerase and genomic DNA as the template. PCR amplification under these conditions is shown to generate 2 to 3 mutations per 1.5-kb of DNA generated (Guo *et al.*, 1999; Zhou *et al.*, 2003). The PCR amplified fragments were then digested with *Nde*I and *Xba*I, cloned downstream of the pMV306 plasmid bearing the *dnaA* promoter, transformed into *E. coli* Top10 (Invitrogen Inc.) and was selected for kanamycin resistance. All transformants were pooled, the plasmid DNA was purified and electrotransformed into *M. tuberculosis dnaA* strain carrying the integrated copy of *dnaA* (expressing from its native promoter) at the *attB* site (Madiraju *et al.*, 2006). Mycobacterial plasmids integrated at the *attB* site can be excised with an incoming target plasmid carrying an alternate antibiotic marker (Pashley and Parish, 2003). Next, all kanamycin resistant and viable transformants were replica patched, incubated at both 37°C and 30°C. Those colonies that grew at 37°C but not at 30°C were identified, subsequently colony purified and are designated as *dnaA* cold sensitive mutants (*dnaAcos*).

Mapping and nucleotide sequencing

Genomic DNA was prepared from *dnaAcos* strains grown at the permissive temperature (37°C), the *dnaA* coding region was amplified with proof reading DeepVent DNA polymerase and sequenced to map mutations.

DnaA overproduction and purification

Purification of the recombinant wildtype *M. tuberculosis* DnaA from *E. coli* BL21(DE3)/pLysS cells on nickel affinity columns was performed as described (Madiraju *et al.*, 2006; Yamamoto *et al.*, 2002a). The coding region of *dnaAcos115* was amplified, cloned downstream of the T7 promoter in a pET19b vector previously digested with *Nde*I and *Bam*HI enzymes. The recombinant mutagenic protein was purified using the same protocol as described for the wild-type DnaA protein.

Filter binding experiments

A nitrocellulose filter binding assay for detecting the binding of ATP to DnaA protein was performed similarly to that described previously (Yamamoto *et al.*, 2002a). Reactions were carried out in 50 µl of buffer containing 50 mM Tris-acetate (pH 8.2), 0.05 mM magnesium acetate, 0.3 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM ammonium sulphate, 20% glycerol, and 0.005% (v/v) Tween-20 was incubated with DnaA and [α -³²P]-labelled ATP. DnaA was also incubated with ATP at the indicated temperature, samples were filtered, washed, and the amount of bound radioactivity was measured by using scintillation counting.

RNA extraction and quantitative real-time (QRT) PCR

Extraction of total RNA from broth-grown cultures of *M. tuberculosis* were performed at different times (see Fig. 3A) and at different temperatures as described previously (Chauhan *et al.*, 2006b; Fol *et al.*, 2006). DNA contamination was removed by treatment with DNaseI

(Ambion). Approximately 50–100 ng of total RNA was reverse-transcribed using 100 nM of RT16S3 with RTdnaA primers and Superscript II reverse transcriptase (Invitrogen). Target cDNA from the control and the experimental sets were amplified in separate reaction tubes. Real-time PCR (Taqman chemistry) was performed in a Bio-Rad iCycler using the Taq DNA polymerase (NEB), Taqman probes (Biosearch Technologies), and reverse and forward primers (see Table 1). The calculated threshold cycle (Ct) value for each gene of interest was normalized to the Ct value for the 16S and the fold-expression was calculated using the formula: fold change = $2^{-\Delta(\Delta C_t)}$. No reverse transcriptase (RT) reactions were included as negative controls. Expression data are the average from three independent RNA preparations, each reverse-transcribed and quantified by real-time PCR in triplicate. Real-time PCR conditions include initial denaturation at 95°C for 3 min; followed by 40 cycles of denaturation at 95°C for 30 sec, annealing and extension at 60°C for 1 min.

DNA synthesis: Incorporation of ^3H Uracil

A quantitative measurement of DNA synthesis with *M. tuberculosis* cultures was performed as described previously (Greendyke *et al.*, 2002) by following Wayne's protocols (Wayne, 1977). To increase the specific activity, all cultures were pre-labelled by growing with uracil (0.5 $\mu\text{Ci/mL}$) for several generations until the OD₆₀₀ reached approximately 0.6. The pre-labelled cultures were then diluted into fresh 7H9 Middlebrook broth containing ^3H -uracil, grown at 37°C for 24h (~ 1 generation). One half of the sample was shifted to 30°C and the other half of the sample was continued at 37°C for 26h. DNA synthesis at both temperatures was then followed at the indicated time periods. In some experiments, the cultures grown at 30°C for 26h were returned to 37°C for 2h. At this point these cultures were either incubated at 37°C or were shifted back to 30°C. Samples consisting of 0.5 ml aliquots were collected for monitoring DNA synthesis at the time points indicated. After incubation with 0.2N KOH at 37°C for 24h, paraformaldehyde was added to a final concentration of 4% and incubated at 37°C for 1h. The samples were then filtered through a 0.45 micron pore size filter, washed twice with phosphate buffered saline followed by 70% ethanol. The membranes were dried and radioactivity counted in a scintillation counter.

Microscopy

M. tuberculosis wildtype and *dnaAcos* strains were grown under various conditions, fixed in 4% paraformaldehyde and visualized using microscopy as previously described (Chauhan *et al.*, 2006a; Chauhan *et al.*, 2006b). Nucleoids were visualized by staining with a mixture of ethidium bromide (40 $\mu\text{g mL}^{-1}$) and mithramycin A (180 $\mu\text{g mL}^{-1}$) for 30 min on ice. Prior to staining, cells were exposed to 2 % (v/v) toluene for 2 min. A Nikon Eclipse 600 microscope with a 100 $\times$ Nikon Plan fluor oil immersion objective, a numerical aperture (NA) of 1.3, a 100 W mercury lamp, and a Chroma filter set (excitation from 540 to 565 nm and emission from 560 to 623 nm) was used for microscopy. Images were acquired using a Photometrics Coolsnap ES camera and Metamorph 6.2 imaging software (Universal Imaging Corporation).

Immunoblotting

Preparation of cellular lysates and immunoblotting using anti-DnaA and anti-SigA antibodies were performed essentially as previously described (Chauhan *et al.*, 2006a; Chauhan *et al.*, 2006b). The SigA protein was used to normalize for protein amounts loaded in each lane when comparing the DnaA levels. Anti-sigma 70 antibodies were obtained from Neoclone Biotechnology (Madison, WI) and used as recommended. Immunoblots were processed with the ECF Western blotting kit from GE Healthcare and scanned on a Bio-Rad Molecular Imager (FX), and DnaA and SigA levels were determined with the volume analysis function of the QuantityOne software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization of *dnaA* mutants: (A): Replica patching of *M. tuberculosis* *dnaA* mutants at 37 and 30 °C. Agar plates were incubated at respective temperatures and visualized after 2 weeks for 37°C and 4 weeks for 30°C. Note that some patches that grew at 37°C did not grow at 30°C. These are potential cold-sensitive mutants. (B): Recovery of cold-sensitive colonies. Plates growing at 30°C were transferred to 37°C and incubated for three weeks. Note colonies that were cold-sensitive at 30°C recovered when returned to 37°C. (C) Mapping of *dnaA* mutants. Nucleotide sequencing of the *dnaA* coding region of *dnaA* mutants located mutations. Mutated residues and the affected DnaA regions are indicated. (D) Viability of *M. tuberculosis* *dnaA* mutants. Actively growing *dnaA* cultures were serially diluted, spread on 7H10 Middlebrook agar plates, incubated at 37°C and 30°C and viable colonies obtained after 4 weeks of incubation were counted from three separate experiments. Hatched bar represents 37°C whereas light bar 30°C. (E) Cell morphology of the *dnaA*115 strain at different temperatures. The *dnaA*115 cells growing at 37°C were shifted to 30°C and incubated for two days prior to returning to 37°C. Incubations were continued for 24h. Samples at different periods of incubation were removed and visualized by brightfield microscopy. Although not shown, the cell morphology phenotype of other strains was similar to *dnaA*115.

Figure 2.

DNA synthesis profiles of the *dnaAcos* cultures at 37°C and 30°C. Actively growing cultures of *M. tuberculosis* wildtype and *dnaAcos* strains were diluted and pre-labelled with ^3H -uracil (0.5 microCurie/mL) and grown to O.D600=0.6. The pre-labelled cultures were diluted into fresh broth containing ^3H -uracil (0.5 $\mu\text{Ci/mL}$) and divided into two parts. One culture was kept at 37°C whereas the other at 30°C. DNA synthesis at indicated time periods was followed. (A): DNA synthesis of cultures growing at 37°C and (B) at 30°C.

Figure 3.

Synchronized DNA replication in *M. tuberculosis* *dnaAcos* cultures. (A) Experimental plan. Two identical sets of *M. tuberculosis* cultures, one indicated by dotted line and the other by solid line, were pre-labelled with ^3H -uracil (0.5 $\mu\text{Ci/mL}$). Both sets of cultures were transferred to 30°C (time point marked as I) and incubated for 26h and shifted to 37°C (time point marked as II). After 2h incubation at 37°C, cultures shown as dotted line were kept at 37°C, whereas the one shown with solid line was returned to 30°C (time point marked as III). A second temperature shift at indicated time period was carried out for cultures growing at 30°C (solid line) (time point marked as IV). Again these cultures were incubated at 37°C for 2 h and then returned to 30°C (time point marked as V). (B) DNA synthesis of cultures

grown at 30°C: At indicated time periods after the temperature shift, samples were removed and processed for determination of radioactivity. (C) Same as above except that DNA synthesis was followed at 37°C. Dotted lines represent extrapolated DNA synthesis pattern. Calculation of the rates of DNA replication at both temperatures. The time required to complete one round of DNA synthesis is calculated to be approximately 11h (660 min) at 30°C and 10h (600 min) at 37°C. *M. tuberculosis* genome size is 4 MB (4000000 bases). Assuming that the DNA synthesis is bi-directional, then the calculated rate of DNA synthesis is expected to be approximately 2 MB per 11h or 3030 nucleotides per min at 30°C and 2MB per 10h or 3333 nucleotides per min at 37°C.

Figure 4.

Expression levels of the *dnaA* and the *mtrA* genes by quantitative real-time (QRT) PCR: RNA was isolated from wild type *M. tuberculosis* and *dnaA*cos strains growing at 37°C (marked as I) prior to temperature shift, 26h after incubation at 30°C (marked as 0) and 4, 8, 12 and 16 h after temperature shift were prepared. The expression levels of the *dnaA* (A), and the *mtrA* (B) mRNA were measured by QRT-PCR using Taqman chemistry. Nucleotide sequences of Taqman probes and oligonucleotides used are listed in Table 1. Relative expression levels at different time points with respect to “0” time point was plotted. The expression levels of *dnaA*, and *mtrA* mRNA were normalized to 16s mRNA levels. Mean $\pm$ SD from three independent experiments are shown.

Figure 5.

DnaA protein levels in *dnaAcos* strains: (A): Actively growing wildtype and *dnaAcos* cultures at 37°C were shifted to 30°C and incubated for 48h. Samples at both temperatures were removed, clear cellular lysates were prepared and the DnaA levels were compared by immunoblotting (panel i). The same blot was also probed with anti-SigA antibodies as described (panel ii) (Chauhan *et al.*, 2006b). (A) Wildtype and *dnaA*₁₁₅, (B) wildtype and *dnaA*₂₃₁, (C) wildtype and *dnaA*₂₄₅. Panel ii: Nitrocellulose blots were scanned on a Bio-Rad Molecular imager (FX) and the levels of DnaA and SigA were determined with volume analysis function of the QuantityOne software. The ratios of DnaA : SigA protein in different strains at 37 and 30 °C are presented.

Figure 6.

Binding of Wild type DnaA and DnaA115 proteins to ATP. Nitrocellulose filter binding assay was used to measure the binding of wildtype DnaA (A) or DnaA115 (B) to radiolabelled ATP. Concentrations of ATP used are shown. The amount of ATP bound (nmoles) per DnaA molecule (pmoles) at 37°C are shown as filled squares and at 30°C as filled triangles. All experimental conditions were as described under experimental procedures.

Figure 7. Visualization of the cell and nucleoid morphology of the wildtype and the *dnaA115* strains. *M. tuberculosis* cultures growing at 37°C and 30°C were removed, stained with ethidium bromide and mithramycinA mixture and visualized by bright-field (panels marked as 'I') and fluorescent microscopy (panels marked as 'ii'). The bar represents "10 μ m".

Table 1

Primers for RT and QRT-PCR.

Oligo name	Oligo sequence	Description
MVM9 TB	5'-AGCTCACGACACAGGTACAC-3'	RT primer for dnaA gene
RT16S3	5'-CCGCACGCTCACAG-3'	RT for 16S rRNA
MVM572mtrrt	5'-GTCAGCACCACAGTCGGGTTCTCGG-3'	RT for MtrA gene
RT16S1	5'-GAGTGGCGAACGGGTGAGTAACA-3'	Forward primer for 16S rRNA
RT16S2	5'-CACCCACCAACAAGCTGATAGG-3'	Reverse primer for 16S rRNA
TP16S	5'-FAM-d(TCCACCACAAGACATGCATCCCGTG)-BHQ-3'	Taqman probe for 16S rRNA
RTdnaA1	5'-AGTCCGGTCTCGGCAAGAC-3'	Forward primer for dnaA gene
RTdnaA2	5'-TGAAGTCGTTGGTGAATTCCTC-3'	Reverse primer for dnaA gene
TPdnaA	5'-FAM-d(TGCCCAACGGTTGTTCCCGG)-BHQ-3'	Taqman probe for dnaA gene
MtrAF	5'-GTGGTGCTGGGTCTGGAGTCG-3'	Forward primer for MtrA gene
MtrAR	5'-CTTGTCGCCGGTACGTCGATTC-3'	Reverse primer for MtrA gene
MtrATP	5'-FAM-d(CGCGCATGGACAGCATCTCGGCGGGTTCG)-BHQ-3'	Taqman probe for MtrA gene