ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 12, pp. 1320-1327. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 12, pp. 1622-1630.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM09-089, July 12, 2009.

Characterization of Physical Interaction between Replication Initiator Protein DnaA and Replicative Helicase from *Mycobacterium tuberculosis* H37Rv

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Received March 17, 2009
Revision received May 1, 2009

Abstract—In the pathogenic *Mycobacterium tuberculosis* H37Rv, the causative agent of tuberculosis, the genetic and biochemical mechanisms for initiation of DNA replication are largely unknown. In the present study, we have characterized the physical interactions between *M. tuberculosis* DnaA and DnaB using both *in vivo* methods, such as bacterial two-hybrid assays, and *in vitro* techniques, such as surface plasmon resonance (SPR) and Pull-down/Western blotting. The full-length N-terminus (1-206 residues) of DnaB was found to interact with DnaA, while the shorter N-terminal domain of DnaB (1-125 residues), which lacked the linker region, did not. Further SPR and electrophoretic mobility shift assays indicated that the N-terminus (1-206 residues) of DnaB also had a critical role in regulating DnaA complex formation at the origin of replication (OriC). This regulatory effect was not obviously observed for DNA substrates containing only two DnaA-boxes. This is the first report showing a physical interaction between DnaA and replicative helicase DnaB from *M. tuberculosis* and the role in subsequent DnaA–OriC interactions. The findings reported here further the understanding of the regulatory mechanisms for initiation of DNA replication in this important human pathogen.

DOI: 10.1134/S0006297909120049

Key words: *Mycobacterium tuberculosis*, DnaB, replication origin, DnaA, OriC

DNA replication is one of the most important biological processes in living organisms and must be precisely coordinated to ensure accurate genome inheritance [1, 2]. In eubacterial chromosomes, a single origin of replication (OriC) is recognized by the initiator protein DnaA, which usually interact with each other to form circular oligomers. The DnaA protein acts by searching for OriC and binding to the DnaA box in this region, thereby distorting the DNA to expose a single-stranded template for the recruitment of the helicase, DnaB [3, 4].

Contact between the replicative helicase and the initiator is an absolute requirement for replication initiation as indicated by studies of protein–protein interactions between the DnaA and DnaB in *Escherichia coli* and *Thermus aquaticus* [5-7]. However, there is very little information available about the dynamics of these interactions, especially in important human bacterial pathogens such as *Mycobacterium tuberculosis* H37Rv, the causative agent of tuberculosis and a major cause of death worldwide. Previous studies have indicated that the organization of the *M. tuberculosis* OriC sequence is complex and the number, distribution, and orientation of DnaA boxes is quite different from that of other bacterial counterparts [8-10]. In contrast to what is observed in *E. coli*, ATPase activity of *M. tuberculosis* DnaA promoted rapid oligomerization on OriC [11]. The full-length DnaB protein also contains an intein and although it has proven difficult to purify, the N-terminal region of DnaB has been successfully expressed and its crystal structure determined [12]. A further difference is reflected in the unique growth characteristics of *M. tuberculosis*, which can maintain an unusual non-replicative persistent state for extended periods *in vitro* and *in vivo* [13, 14]. These unique characteristics suggest that *M. tuberculosis* might possess alternate mechanisms for DNA replication that

Abbreviations: 3-AT, 3-amino-1,2,4-triazole; GST, glutathione-S-transferase; NTA-chip, nitrilotriacetic acid chip; OriC, origin of replication; PCR, polymerase chain reaction; SA-chip, streptavidin chip; SPR, surface plasmon resonance; str, streptomycin.

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allow it to adapt to the multiple stresses it encounters during the infection process [15].

Most of our knowledge of replisome assembly mechanisms and interactions comes from studies in *E. coli* [5, 7]. However, despite decades of research, many aspects still remain poorly understood due to the great complexity of this system. Very little is known about these processes in actinomyces, including clinically important species such as *M. tuberculosis*. In the present study, we have provided the first documentation of physical interactions between DnaA and replicative helicase DnaB from *M. tuberculosis*.

MATERIALS AND METHODS

DNA and oligonucleotides. *Mycobacterium tuberculosis* genomic DNA and specific primers were synthesized by Invitrogen (USA) (as shown in Table 1). The coding regions of *M. tuberculosis* genes were amplified by PCR (polymerase chain reaction) using these primers. Plasmids and DNA fragments were purified using kits according to the manufacturer's protocols (Watson, USA).

Cloning, expression, and purification of recombinant proteins. *Mycobacterium tuberculosis* genes and their mutants were amplified from genomic DNA using their special primers and were cloned into overexpression vectors of pET28a or pGEX-4T-1 (Table 2). The 6×His-tag proteins and glutathione-S-transferase (GST) proteins containing N-terminal tags were purified according to previously published procedures [12]. These recombinant proteins with different affinity tags were then used to analyze protein–protein interactions. Protein concentrations were determined according to previously published procedures [16]. All purified proteins were greater than 95% pure as determined by SDS-PAGE and subsequent staining by Coomassie Blue.

Bacterial two-hybrid analysis. A BacterioMatch II Two-Hybrid System Library Construction Kit (Stratagene, USA) was used to detect protein–protein interactions between *M. tuberculosis* DnaA and DnaB proteins. The experiment was carried out according to the manufacturer's instructions. Recombinant pBT and pTRG vectors containing *M. tuberculosis* genes and their different domains were generated (Table 2). Positive growth cotransformants were selected on the Selective Screening Medium plate containing 5 mM 3-amino-1,2,4-triazole (3-AT) (Stratagene), 8 μg/ml streptomycin (str), 15 μg/ml tetracycline, 34 μg/ml chloramphenicol, and 50 μg/ml kanamycin. A cotransformant containing pBT-LGF2 and pTRG-Gal11P (Stratagene) was used as a positive control for expected growth on the Selective Screening Medium. A cotransformant containing empty vector pBT and pTRG was also used as a negative control.

Surface plasmon resonance (SPR) analysis. The interactions of *M. tuberculosis* DnaA proteins with DnaB N-terminal domains were analyzed on a BIAcore 3000 instrument (GE Healthcare, USA) according to previously published procedures [16, 17]. The assays were per-

Table 1. Primers used in this study

Name	Sequence (5' → 3')*
DnaA-fw	ATATAGCGGCCGCATTGACCGATGACCCCGG
DnaA-rv	GTGGTCTAGACTAGCGCTTGGAGCGCTGACG
DnaAN-rv	GTGGTCTAGAGAACCGGTTGGAGGCGCCGAT
DnaAC-fw	TATATGCGGCCGCAGCGCACGCCGCCGCCTT
DnaB-fw	GCGAGAATTCATATGGCGGTCGTTGATGACCTAG
DnaB-rv	ATATTCTAGATCAACCGAGCCATGTTGGCGAAGCG
DnaBN1-fw	GCAGGAATTCCAATGGCGGTCGTTGATGACCTA
DnaBN1-rv	ATATTCTAGATCATTCGGCAACGATGCTCGCGT
DnaBN2-fw	TATAGAATTCATAAGGCGCTGCTGCGCCGGCTG
DnaBN3-rv	TATATCTAGATAAGAAGCCGGTAGCCACCCCGC
DnaBC delta 59-fw	ATAGGAATTCCTATGACCGAACTCGACGAGGTC
DnaBC delta 59-rv	ATATATCTAGATCAGCCCGATTCCCTGAGGTCG
MtOriC-fw	biotin-TACAGAATTCCACGGCGTGTTCTTCCGACAACGT
MtOriC-rv	TATCTCTCTAGATGCGCCCTTTCACCTCACGATGAG
Dbox-fw	biotin-AGACACTTGTCCACAGGCTGTGCACAACAACTT
Dbox-rv	AAGTTGTTGTGCACAGCCTGTGGACAAGTGTCT

* Sites for restriction enzymes are underlined.

Table 2. Plasmids and recombinant vectors used in this study

Plasmid	Description
pTRGDnaA	pTRG derivative for bacterial two-hybrid assay
pTRGDnaAN	pTRG derivative for bacterial two-hybrid assay
pTRGDnaAC	pTRG derivative for bacterial two-hybrid assay
pBTDnaB	pBT derivative for bacterial two-hybrid assay
pBTDnaBN1	pBT derivative for bacterial two-hybrid assay
pBTDnaBN2	pBT derivative for bacterial two-hybrid assay
pBTDnaBN3	pBT derivative for bacterial two-hybrid assay
pBTDnaBC delta 59	pBT derivative for bacterial two-hybrid assay
pBTDnaB delta 59	pBT derivative for bacterial two-hybrid assay
pET28aDnaA	pET28a derivative for expression 6His-DnaA
pET28aDnaBN1	pET28a derivative for expression 6His-DnaBN1
pET28aDnaBN3	pET28a derivative for expression 6His-DnaBN3
pGEX4T1DnaBN1	pGEX-4T-1 derivative for expression GST-DnaBN1
pGEX4T1DnaBN3	pGEX-4T-1 derivative for expression GST-DnaBN3
pTRG*	bacterial two-hybrid assay target domain vector
pBT*	bacterial two-hybrid assay bait domain vector
pET28a**	Kan^r expression vector with 6His-tag coding sequence
pGEX-4T-1***	Amp^r expression vector with GST-tag coding sequence

* From Stratagene, USA.
** From Novagen, USA.
*** From GE Healthcare, USA.

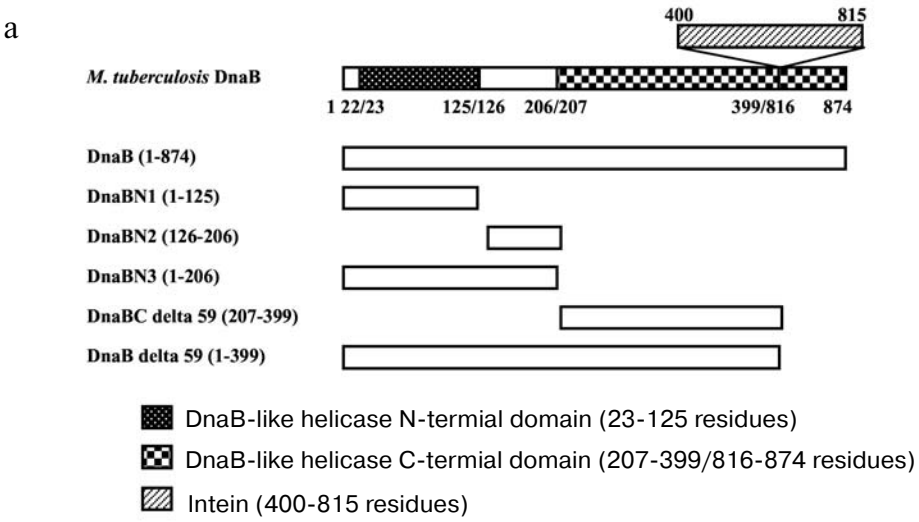
formed at 25°C. The DnaA protein was immobilized onto the NTA chips (nitrilotriacetic acid chip). The purified DnaB protein, to be used as the ligand, was diluted in HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 50 μM EDTA, 5 mM ATP, 0.005% BIAcore surfactant P20) to a concentration of <200 nM and injected at 10 μl/min for 5 min. For a negative control, the GST protein was substituted for the DnaB protein. Each analysis was performed in triplicate. An overlay plot was produced to depict the interaction between replication proteins. The interaction between the origin DNA and the replication proteins was also assayed by SPR using biotin-labeled promoter DNA immobilized onto the SA chips (streptavidin chip) (GE Healthcare). Each analysis was performed in triplicate. An overlay plot was produced to distinguish the interactions between replication proteins.

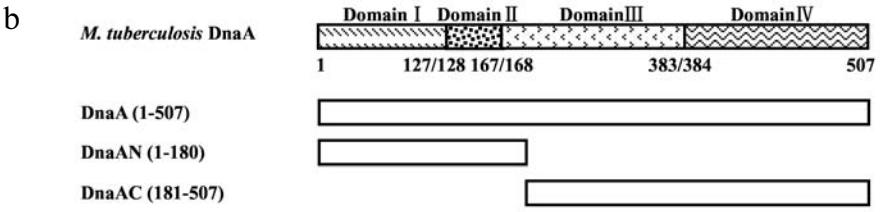
Pull-down and Western blotting analysis. Equimolar amounts of either normalized GST, GST-DnaBN1, or GST-DnaBN3 proteins were combined with the normalized 6×His-DnaA in 1.5 ml tubes containing 10% Ni-NTA agarose (Qiagen, Germany) in 1 ml TN buffer (20 mM Tris, pH 7.9, 150 mM NaCl). The beads were washed twice with 1 ml TN buffer containing 5 mM imidazole and centrifuged at 3500 rpm for 1 min. Proteins bound to the beads were eluted with 100 μl TN buffer containing 200 mM imidazole. The proteins were then separated on 10% SDS-polyacrylamide gel and further analyzed by Western blotting using anti-6×His antibodies.

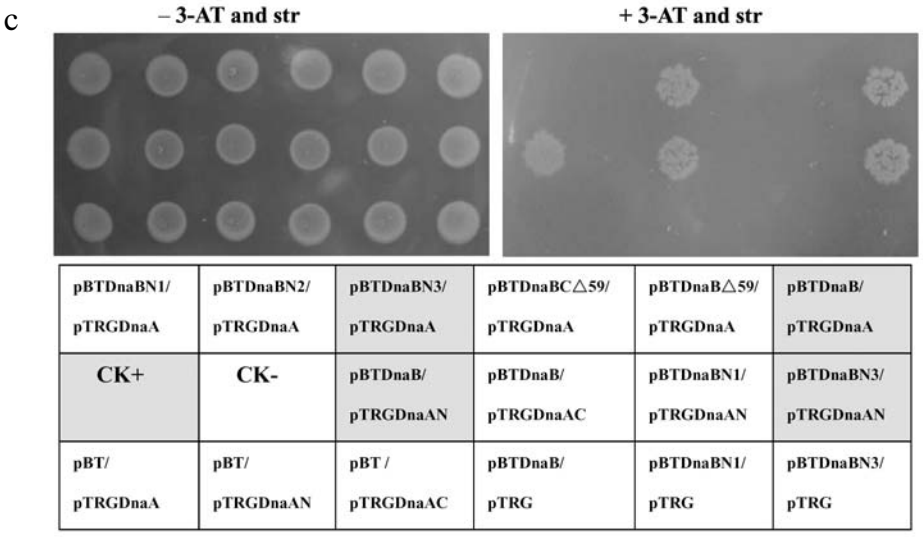
RESULTS

Design of DnaA and DnaB mutants and bacterial two-hybrid assays for protein–protein interaction studies. A previous study [12] showed that *M. tuberculosis* DnaBN (residues 21-197 including the linker region) formed a stable dimer in solution and a hexamer in a crystallization solution. In contrast, a truncated form of the protein consisting only of residues 21-134 was monomeric. Based on these findings and using conserved sequence motifs [3, 10, 12], several DnaB mutants (designated DnaBN1, DnaBN2, and DnaBN3) were designed for the current study and were amplified using specific PCR primers (Table 1 and Fig. 1a). Several DnaA mutants were also designed according to the conserved sequence motifs (Fig. 1b). These DnaA and DnaB fragments were further cloned into bacterial two-hybrid vectors and over-expression plasmids (Table 2).

The putative interaction domain(s) of the *M. tuberculosis* DnaB helicase with the initiator protein DnaA were then studied by assaying the interactions between different domains of DnaA and DnaB using bacterial







pBTDnaBN1/ pTRGDnaA	pBTDnaBN2/ pTRGDnaA	pBTDnaBN3/ pTRGDnaA	pBTDnaBC△59/ pTRGDnaA	pBTDnaB△59/ pTRGDnaA	pBTDnaB/ pTRGDnaA
CK+	CK-	pBTDnaB/ pTRGDnaAN	pBTDnaB/ pTRGDnaAC	pBTDnaBN1/ pTRGDnaAN	pBTDnaBN3/ pTRGDnaAN
pBT/ pTRGDnaA	pBT/ pTRGDnaAN	pBT / pTRGDnaAC	pBTDnaB/ pTRG	pBTDnaBN1/ pTRG	pBTDnaBN3/ pTRG

Fig. 1. Schematic representation of *M. tuberculosis* DnaB and DnaA and two-hybrid assays for their interactions. a) Schematic diagrams of the full-length and the different deletion DnaB mutants. The names given to mutants are listed to the left of the panel. The amino acid regions that have been deleted are also shown. b) Schematic diagrams of full-length and different deletion DnaA mutants. The gene contains four domains each indicated by a different frame. The second domain is a linker. c) Two-hybrid assays for the interactions between the *M. tuberculosis* DnaA and DnaB proteins. Left panel, plate minus streptomycin (str) and 5 mM 3-amino-1,2, 4-triazole (3-AT). Right panel, plate plus str and 5 mM 3-AT. An outline of the plates: CK+, co-transformant containing pBT-LGF2 and pTRG-Gal11P as a positive control; CK–, co-transformant containing pBT and pTRG as a negative control. Each unit represents the corresponding co-transformant in the plates, and the units with tinged background represent the co-transformants that could grow well on the screening medium. Growth of co-transformants was observed in the shadowed boxes.

two-hybrid systems (Stratagene). As shown in Fig. 1c, a positive co-transformant grew on a Selective Screening Medium, but the corresponding negative co-transformant did not grow at all. Interactions between DnaB and DnaA, DnaBN3 and DnaA, DnaB and DnaAN, and DnaBN3 and DnaAN were apparent (Fig. 1c). The region spanning residues 125-206 appeared to be required for the interaction, as the co-transformants containing truncated forms missing these residues (DnaBN1 or DnaBN2) did not grow (Fig. 1c). Therefore, the full-length N-terminus of DnaB appeared to be essential for its interaction with DnaA.

Physical interaction between DnaA and DnaB. Using purified 6×His-tagged and GST-tagged recombinant proteins, the interactions between DnaA and two different DnaB N-terminal fragments (DnaBN1 and DnaBN3) were characterized by a SPR assay in the presence of 5 mM ATP. The 6×His-DnaA protein was immobilized onto an NTA chip. When 400 nM DnaBN3 was passed over the chip, a significant response of approximately 110 RU was observed. No response was obtained for 400 nM DnaBN1, which lacks the additional linker sequence, nor was a response observed for either the control GST protein or for the running buffer alone (Fig. 2a).

A Pull-down/Western blotting assay was used to further characterize the interaction of DnaA and DnaB by co-incubating 6×His-DnaA protein with GST-DnaBN1, GST-DnaBN3, or GST. When the solutions were analyzed by 10% SDS-PAGE and Western blotting using anti-6×His antibody, as shown in Fig. 2b, an obvious hybridization signal was observed in the eluate only for GST-DnaBN3. No signal was observed for either GST-DnaBN1, which lacked the linker region, or for GST alone.

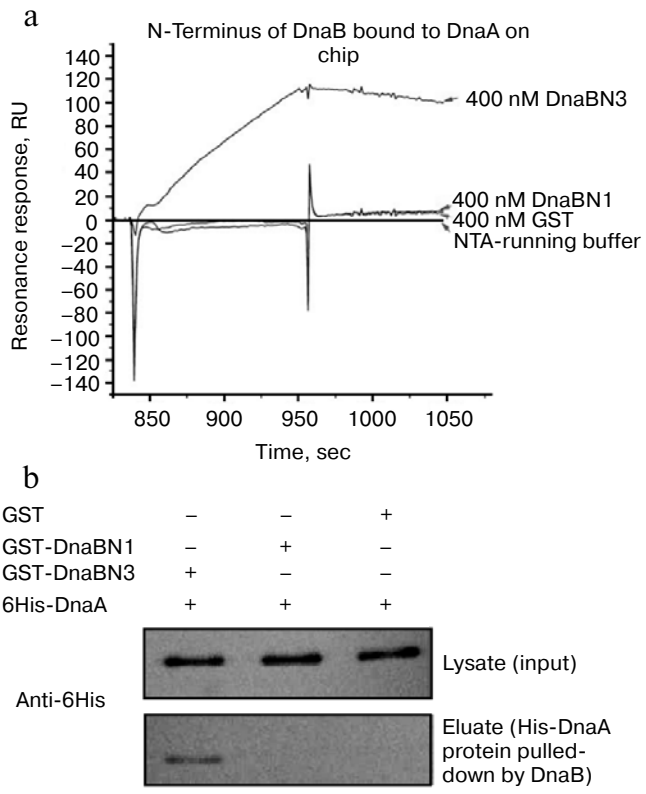


Fig. 2. Physical interactions between the N-terminus of *M. tuberculosis* DnaB and DnaA proteins. a) The interaction between DnaB and DnaA was monitored using SPR on a BIAcore 3000 [16]. 6×His-tag DnaA protein (5 nmol) was immobilized on the chip surface and DnaB was passed over the chip and then allowed to dissociate for 10 min. An overlay plot depicting the interaction of DnaA with DnaBN1 and DnaBN3 was produced. GST protein and HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 50 μM EDTA, 5 mM ATP, 0.005% BIAcore surfactant P20) were used as negative controls. b) Pull-down/Western blotting assays for examining the specific interaction between the N-terminus of DnaB and DnaA. The proteins were purified for this assay. Equimolar amounts of 6×His-DnaA combined with GST-DnaBN3 were used for pull-down assays as described in "Materials and Methods". GST or GST-DnaBN1 was used as control. All samples taken before and after GST pull-down were detected by immunoblotting using anti-6×His antibody.

The N-terminus of DnaB regulates formation of a DnaA–OriC complex on chips. The effect of *M. tuberculosis* DnaB on the binding activity of DnaA with the DNA origin of replication (OriC) was examined using a full-length DNA fragment of OriC, which was amplified and biotinylated at its 5-terminus for immobilization onto an SA chip (Fig. 3a). Using the SPR technique, we assayed the interaction of the DnaA protein with DNA OriC. The modulating effects of the two mutations, DnaBN1 and DnaBN3, on these interactions were also studied.

When 125 nM DnaA was passed over the OriC-immobilized SA chip, a significant association response of about 180 RU was observed. In contrast, running buffer gave no response (Fig. 3b). A greatly decreased response (100 RU) was observed when 200 nM DnaBN3 was incubated with the 125 nM DnaA protein prior to passage over the chip (Fig. 3b). This inhibition was more pronounced if the concentration of DnaBN3 was increased to 350 nM. In contrast, only a slight influence was observed when 350 nM DnaBN1 was passed over the chip together with 125 nM DnaA (Fig. 3b). In addition, no response was observed when either DnaBN1 or DnaBN3 alone was passed over the OriC chip, which demonstrated that these proteins themselves did not bind with the DNA on the chip.

The N-terminus of DnaB does not influence interaction of DnaA protein with DnaA-box DNA. Eubacterial DnaA molecules contain the two-domain AAA^+ motif and usually interact with each other to form circular oligomers [3, 4]. This ability of DnaA allows for the formation of large complexes with OriC, which often contain multiple DnaA boxes. In the above study, where DnaBN3 was shown to inhibit this DnaA–OriC complex formation, the inhibition might have been a result of interference with the formation of DnaA multimers, rather than a direct regulation of the interaction of DnaA

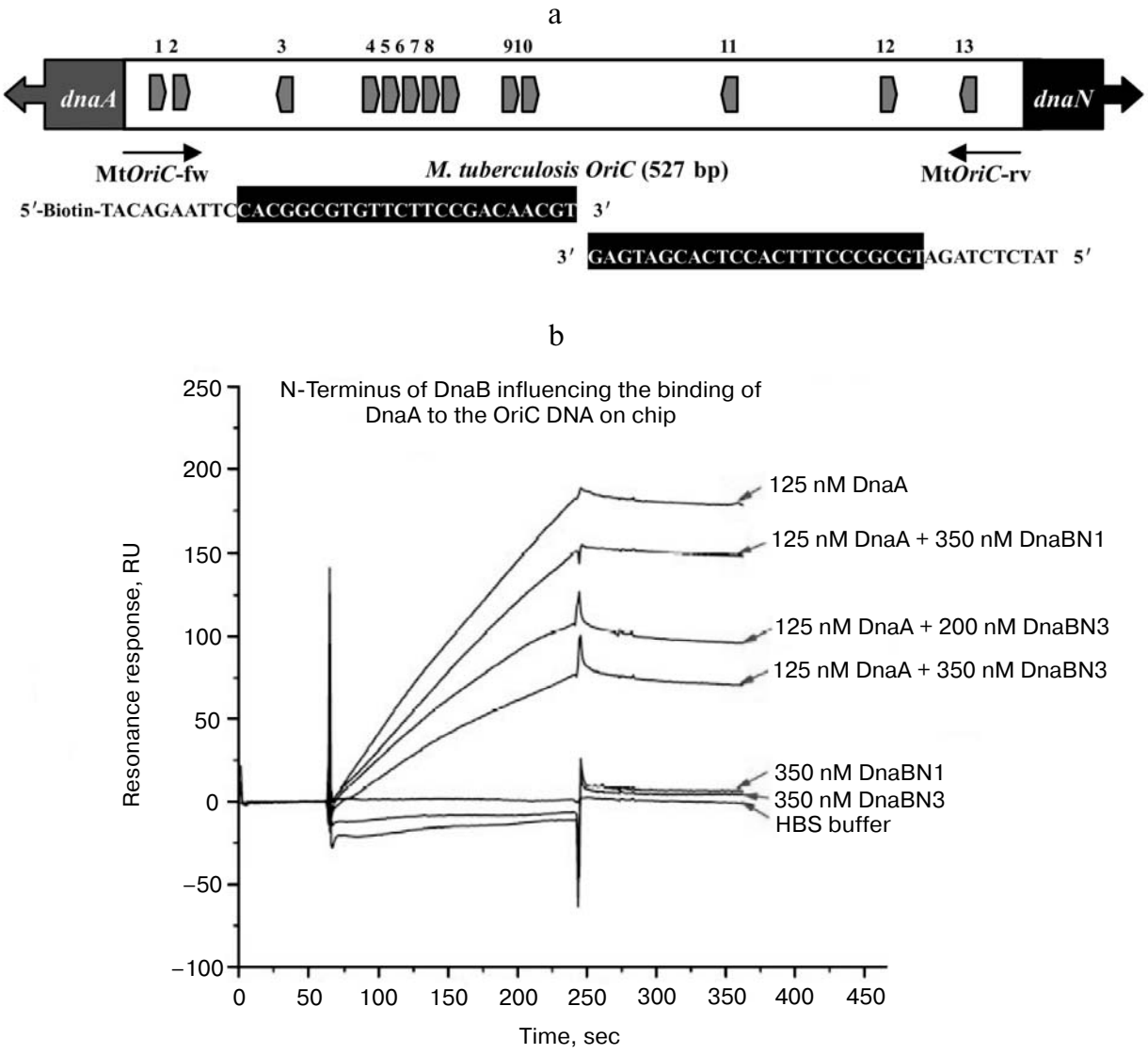


Fig. 3. SPR and EMSA analysis of the interaction between *M. tuberculosis* OriC and DnaA. a) The structure of *M. tuberculosis* OriC. The origin region contains 13 DnaA boxes [11] of approximately 527 bp length. The complementary sequences with OriC in the primers are displayed as black background and white characters. b) The interaction between OriC and DnaA as monitored using SPR on a BIAcore 3000. Biotinylated OriC DNA was immobilized on an SA sensor chip. HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 50 μM EDTA, 5 mM ATP, 0.005% BIAcore surfactant P20) was used to produce a baseline. 6×His-DnaA was used as positive control. The plots were produced when the different combinations of DnaA together with DnaBN1 or DnaBN1 alone were passed over the chip.

with OriC by the N-terminus of DnaB. To test this possibility, we designed a short DnaA-box DNA substrate containing just two boxes (Fig. 4a), which reduced the likelihood of complicated DnaA multimeric structures.

When the biotinylated short-DnaA-box DNA substrate was immobilized onto SA chips, no DNA binding was observed when the running buffer, DnaBN1, or DnaBN3 alone were passed over the chip (Fig. 4b). When 80 nM DnaA together with DnaBN3 was passed over the chip, a very slight inhibition of the DNA binding of DnaA was observed. A similar slight inhibition was also observed for DnaA together with DnaBN1 (Fig. 4b). Thus, when a short DNA substrate was used no difference was seen in binding between DnaBN1 and DnaBN3. This was a completely different result from that obtained with OriC (Fig. 3b). A similar result was seen when 150 nM DnaA together with either 250 nM DnaBN1 or DnaBN3 was passed over the chip (Fig. 4b). Therefore, the N-terminus of DnaB had little effect on the DnaA/DnaA-box complex formation.

DISCUSSION

The genetic and biochemical mechanisms of the replication initiation of *M. tuberculosis* are still largely

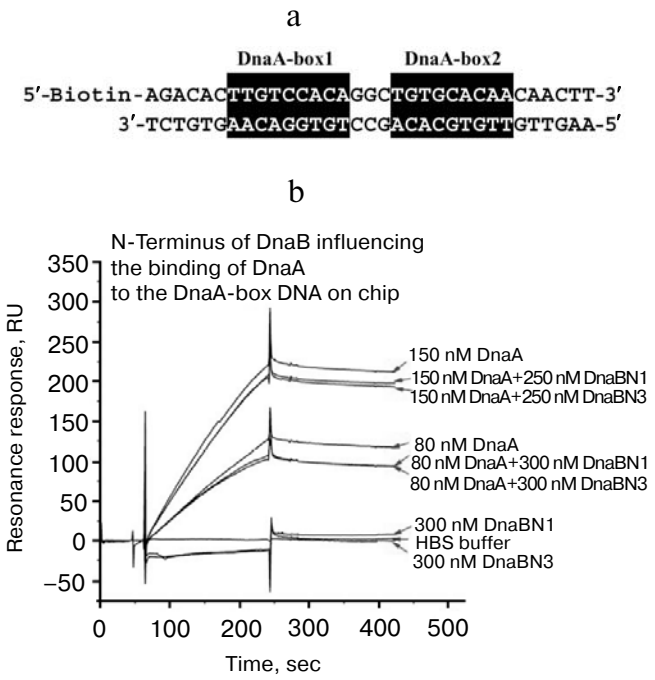


Fig. 4. SPR analysis of interaction between *M. tuberculosis* DnaA box and DnaA. a) The sequence and structure of the DnaA-box-containing oligonucleotides used for this assay. The sequences displayed by a black background are the DnaA-box regions. The oligonucleotide was biotin-labeled at the 5′-terminus and immobilized on an SA sensor chip. b) Effects of different combinations of DnaA with DnaBN1 or only DnaBN1. HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 50 μM EDTA, 5 mM ATP, 0.005% BIAcore surfactant P20) was used to produce a baseline. 6×His-DnaBN1 and 6×His-DnaBN3 had no DNA-binding activities and were used as negative controls. 6×His-DnaA was used as positive control.

unknown. In the current study, the results demonstrate the idea that the N-terminus of DnaB, containing the linker sequence, is essential for the interaction of DnaB and DnaA proteins. Furthermore, the full-length N-terminus of DnaB was shown to modulate DnaA complex formation with OriC. This regulatory effect was not obviously observed for DNA substrates containing only two DnaA-boxes. This is the first report on the interactions of DnaA, DnaB, and OriC from *M. tuberculosis*.

Several studies on the function and interaction of DnaA with DnaB in *E. coli* offer important information about the regulation of bacterial replication initiation, although no similar reports have appeared regarding *M. tuberculosis*. It is known that certain *dnaA* mutants of *E. coli* show a cold-sensitive (Cos) growth phenotype that results from a lethal over-initiation of chromosome replication [18]. Over-initiation and cold sensitivity can be suppressed by partially functional DnaA derivatives as a result of interference with the initiation complex [19]. The Cos phenotype of the dnaA219 (Cos) strain WM2667 has been shown to be suppressed by a peptide that contains amino acid residues localized at the border of the flexible hinge region between the N-terminal domain and the C-terminus of DnaB [5]. The current study on the interaction of DnaB and DnaA in *M. tuberculosis* indicates that the linker region, within N-terminus DnaBN3 of DnaB, is essential for the interaction with DnaA. Meanwhile, this region was found to be essential, but not enough, for the inhibition of DnaA–OriC complex formation. These findings offer an alternative mechanism to explain the suppression of the Cos phenotype of *dnaA* mutants caused by the overexpression of DnaB. The flexible hinge region of DnaB may be a common helicase-initiator interaction domain in both *E. coli* and *M. tuberculosis*.

In previous studies in *E. coli*, the association of DnaA with DnaB has been found to be very weak and required cross-linking to be convincingly demonstrated [5, 6]. In the current study, the interaction between the *M. tuberculosis* DnaA and DnaB was readily detected by the techniques used. This included use of bacterial two-hybrid system, SPR, and even Pull-down/Western blotting assays. Therefore, the interaction between DnaA and DnaB in *M. tuberculosis* appears to be novel and quite different from those in *E. coli*.

Although isolation and purification of the full-length DnaB protein has not yet been achieved in *M. tuberculosis*, its N-terminus containing the linker region was successfully expressed and its crystal structure has been recently resolved [12]. In the current study, we found that it was this N-terminus with the linker region that regulated the interaction between DnaA and DnaB and also modulated DnaA complex formation with OriC. This interaction between DnaA and DnaB in the *M. tuberculosis* appeared to be even stronger than that reported for *E. coli*. Therefore, our findings extend the previous conclusions drawn from results obtained with other prokaryotic organisms, while they offer important new information regarding the regulation mechanisms for initiation of DNA replication in the important human pathogen *M. tuberculosis*.

This work was supported by 973 Program (2006CB504402), the National Natural Science Foundation of China (30930003 and 30670016), and the National Special Key Project of China on Major Infectious Diseases (2008-2010).

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