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Facilitation of Dissociation Reaction of Nucleotides Bound to *Mycobacterium tuberculosis* DnaA

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Acidic phospholipids have been shown to promote dissociation of bound nucleotides from *Mycobacterium tuberculosis* DnaA ($DnaA_{TB}$) purified under denaturing conditions [Yamamoto *et al.*, (2002) Modulation of *Mycobacterium tuberculosis* DnaA protein–adenine-nucleotide interactions by acidic phospholipids. *Biochem. J.*, 363, 305–311]. In the present study, we show that a majority of $DnaA_{TB}$ in non-overproducing cells of *M. tuberculosis* is membrane associated. Estimation of phospholipid phosphorus following chloroform: methanol extraction of soluble $DnaA_{TB}$ purified under native conditions ($nDnaA_{TB}$) confirmed the association with phospholipids. $nDnaA_{TB}$ exhibited weak ATPase activity, and rapidly exchanged ATP for bound ADP in the absence of any added phospholipids. We suggest that the outcome of intra-cellular $DnaA_{TB}$–nucleotide interactions, hence $DnaA_{TB}$ activity, is influenced by phospholipids.

Key words: ADP, ATP, ATPase, DNA replication, mycobacteria.

Abbreviations: *OriC*, origin of replication; Trx, thioredoxin; PEI, polyethyleneimine.

Tuberculosis is one of the most globally prevalent infectious diseases and accounts for ~3 million deaths each year. The causative agent *Mycobacterium tuberculosis*, a Gram-positive bacterium, is a slow grower with approximate doubling time of 24 h. The genus Mycobacterium includes other pathogens such as *M. tuberculosis*, *Mycobacterium bovis* and *Mycobacterium leprae*, and non-pathogens such as *Mycobacterium smegmatis* and *Mycobacterium fortuitum*. The doubling times of these organisms range from 2 to 3 h (*M. smegmatis, M. fortuitum*) to 22–24 h (*M. tuberculosis, M. bovis*) to 185 h (*M. leprae*). The genetic and biochemical factors responsible for the differences in the growth rates of various mycobacteria are largely unknown.

Chromosomal DNA replication in bacteria is regulated at the initiation step, where the activity and quantity of the initiator DnaA protein is critically controlled (*1–3*). DNA replication in *Escherichia coli* is initiated by the binding of DnaA protein to the DnaA boxes in the *oriC*, the origin of chromosomal DNA replication, and these initial interactions result in the melting of the nearby A-T-rich region, thereby forming an open (initiation) complex (*4*, *5*). DnaA protein then recruits the DnaB helicase–DnaC protein complex to form a pre-priming complex, which allows entry of primase and establishment of the replication forks.

Escherichia coli DnaA activity is proposed to be regulated by its binding of adenine nucleotides. DnaA protein has high affinity for ATP and ADP, and the ATP-binding form of DnaA protein (ATP-DnaA) initiates DNA replication *in vitro*, whereas the ADP-binding form (ADP-DnaA) does not (*6*). Membrane phospholipids are believed to play a crucial role in the regulation of DnaA activity (*7*). They affect the dissociation of both ATP and ADP from DnaA and rejuvenate moribund DnaA in to an active species for the *oriC* (*8*, *9*). Acidic phospholipids in a fluid membrane facilitate dissociation of ADP bound to DnaA protein *in vitro*, and the resultant free form of DnaA protein is reactivated through binding to ATP, which is present at high concentrations under physiological conditions.

The key elements involved in the initiation of *M. tuberculosis* DNA replication, namely *oriC* and DnaA, have been identified (*10*, *11*). *Mycobacterium tuberculosis oriC* is 550-bp long (*10*) and recombinant $DnaA_{TB}$ protein purified under denaturing conditions has been shown to associate with adenine nucleotides and *oriC* (*12*). $DnaA_{TB}$ specifically recognizes DnaA boxes and dimethylsulphate footprinting revealed the presence of nine DnaA boxes that bear little or no sequence similarity to the five DnaA boxes of *E. coli oriC* (*13*). Acidic phospholipids have been shown to modulate $DnaA_{TB}$ interactions with adenine nucleotide and *oriC* stabilizes $DnaA_{TB}$–ATP interactions and promotes dissociation of $DnaA_{TB}$–ADP complexes (*12*). Presumably, the interaction of $DnaA_{TB}$ with phospholipids results in a decrease in the affinity of DnaA for ATP, *oriC* or both. Other results suggest that ADP-bound form of DnaA is not competent for binding and rapid oligomerization on *oriC* (*13*). Together, these results suggest that phospholipids regulate nucleotide bound state of DnaA and play important regulatory roles in *M. tuberculosis oriC*

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replication (*12*). To further understand the relationships between $DnaA_{TB}$ and phospholipids, we purified $DnaA_{TB}$ as a soluble protein under native conditions and investigated its interactions with nucleotides in the absence of added phospholipids.

MATERIALS AND METHODS

Purification of M. tuberculosis DnaA Protein—Cloning of *M. tuberculosis DnaA* was performed as described (*12*). Overproduction of the recombinant DnaA protein was carried out with co-producing *E. coli* thioredoxin (Trx) (*14*). For purification, bacterial pellets were collected and re-suspended in binding buffer [20 mM Tris–HCl (pH 8.0), 10% glycerol, 500 mM NaCl, 5 mM 2-mercaptoethanol, 10 mM imidazole and 0.5% Tween-20] and disrupted by sonication. The crude cell lysate was clarified by centrifugation, and the supernatant fraction, which contained the soluble recombinant DnaA protein, was loaded on a Ni^{2+}-nitrilotriacetic acid-agarose column (Qiagen) equilibrated with same buffer. Unless otherwise noted, the purification was done at 4°C. After washing with the same buffer to remove unbound proteins, DnaA protein was eluted with Renaturation buffer [50 mM PIPES (pH 6.8), 10 mM magnesium acetate, 200 mM ammonium sulphate, 20% sucrose, 0.1 mM EDTA and 2 mM 2-mercaptoethanol] with 100 mM imidazole. Protein fractions were analysed by SDS–PAGE (*15*). Fractions containing the eluted DnaA protein were pooled and dialysed against Renaturation buffer. Aggregated protein was removed by centrifugation and the clear supernatant containing soluble DnaA protein was stored at −70°C until use. Preparation of the denatured DnaA protein of *M. tuberculosis* with urea was performed according to our previous paper (*12*).

Nucleotide-Binding Assay—Nucleotide-binding experiments were carried out as explained (*12*). Binding of ATP to DnaA was performed in 100 μl of buffer E [50 mM Tris acetate (pH 8.2), 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM ammonium sulphate, 20% glycerol, 0.005% (v/v) Tween-20] containing various concentrations of [α-^{32}P]ATP and DnaA protein (1 pmol). After incubation at 37°C for 15 min, the reaction mixture was filtered through a nitrocellulose membrane (Millipore HA 0.45 μm, 24 mm diameter) pre-soaked in buffer E. The filter was washed with 10 ml of ice-cold buffer E, dried and the amount of radioactivity retained on the filter was measured in a liquid-scintillation counter. For exchange of ATP with ADP bound to DnaA protein, DnaA protein (2.0 pmol) was incubated with 200 pmol of [^{14}C]ADP in 100 μl of buffer E at 0°C for 15 min, and then with 1.0 mM [α-^{32}P]ATP at 37°C. The reaction mixture was filtered through a nitrocellulose membrane and the amount of radioactivity retained on the filter was measured as described above.

ATPase Activity—ATPase activity of DnaA was measured as described (*12*). Reaction samples were prepared on ice in 40 μl of buffer E containing [α-^{32}P]ATP (100 pmol) and DnaA protein (40 pmol) and transferred to 37°C. Some samples contained the *M. tuberculosis* DnaA and adenine–nucleotide interactions *M. tuberculosis oriC* (0.2 μg). At indicated time intervals, samples were collected on membranes and bound nucleotides were extracted with 50 μl of 1 M formic acid. An aliquot (0.5 μl) of the extracted sample was spotted on the polyethyleneimine (PEI) cellulose (Macherey–Nagel) membranes and subjected to chromatography in 1 M formic acid–0.5 M LiCl. Membranes were dried, spots were visualized in a Molecular Imager (Bio-Rad) and quantified by using the Quantity One program.

Measurement of Organic Phosphate—The concentration of organic phosphate was estimated following Ames and Dublin protocol (*16*). Ten percent of magnesium nitrate (0.03 ml) was added to 0.1 ml of sample in Pyrex 13 × 100-mm test tube. The mixture was taken to dryness and ashed by shaking the tube over a strong flame. The tube was then allowed to cool and 0.3 ml of 0.5 N HCl was added. The material was heated in boiling water for 15 min and cooled to room temperature. A solution measuring 0.7 ml containing 1.3% ascorbic acid and 0.06% ammonium molybdate was added and the tube was incubated at 37°C for 60 min. The colour that has absorbency at 820 nm was read and amount of phosphorous was estimated by comparing with standard prepared using inorganic phosphate.

Western Blot Analysis—Approximately 1 μg protein corresponding to whole-cell lysate, membrane, cell walls and cytosolic fractions of *M. tuberculosis* obtained from Tuberculosis Vaccine Testing and Research Materials, Colorado State University, NIH contract NO1-AI 40091, was resolved by SDS–PAGE, transferred to nitrocellulose membranes and probed with affinity purified anti-$DnaA_{TB}$ antibodies as described (*17*).

RESULTS AND DISCUSSION

Mycobacterium tuberculosis DnaA Protein in Non-overproducing Cells is Membrane Associated—To test association, if any, of $DnaA_{TB}$ protein with phospholipids, we probed whole-cell lysates, soluble cell wall fractions, high-speed membrane and cytosolic fractions prepared from wild-type, DnaA non-overproducing *M. tuberculosis* cells with affinity purified anti-$DnaA_{TB}$ antibodies. As can be seen, a majority of $DnaA_{TB}$ protein in non-overproducing cells is membrane and cell wall associated, but hardly any in the cytosolic fractions (Fig. 1, compare lane 5 with lanes 2–4). These results are consistent with a notion that native $DnaA_{TB}$, like its *E. coli* counterpart, is associated with membranes and cell walls.

Purification of Soluble $DnaA_{TB}$ Under Native Conditions—Membrane association of $DnaA_{TB}$ indicates possible association with phospholipids. To further address this issue, we purified $DnaA_{TB}$ under soluble conditions from *E. coli* cells expressing *E. coli* Trx and *Dna*A genes simultaneously. Co-expression of *trx* promoted the solubility of DnaA, consistent with other report on the increase in the solubility of various foreign proteins with Trx expression (*14*). As can be seen, a distinct band corresponding to DnaA was evident (Fig. 2A). Clear-cell lysates were then used to purify $DnaA_{TB}$ on nickel affinity columns and SDS–PAGE analysis confirmed the purity of the soluble DnaA protein ($nDnaA_{TB}$) (Fig. 2A). Nucleotide-binding experiments revealed that purified $nDnaA_{TB}$ bound ATP in protein

concentration-dependent manner, and that the amount bound was ~5-fold lower than that purified under denaturing conditions (Fig. 2B). Scatchard analysis revealed that K_D for nDnaA$_{TB}$ is ~415 nM (data not shown). This value is higher than those for DnaA$_{TB}$ (69 nM) (*12*) and for *E. coli* DnaA protein (30 nM) (*6*).

DnaA$_{TB}$ is Associated with Phospholipids—Our results showing the association of DnaA with membrane fractions in non-overproducing cells (Fig. 1) and combined with reduced binding of DnaA$_{TB}$ to ATP led us to hypothesize that nDnaA$_{TB}$ is associated with phospholipids. To test this prediction, we extracted nDnaA$_{TB}$ with chloroform: methanol (2:1 v/v), and evaluated the presence of phospholipid phosphorus by colorimetric assay using inorganic phosphate as control. Our results indicate that amount of phospholipids bound to native DnaA was 1.87 pmol phospholipids / 7.4 pmol DnaA protein (data not shown). Since phospholipids promote dissociation of bound nucleotides, we predicted that the biochemical properties of the nDnaA$_{TB}$, *i.e.* ATPase activity and exchange of ATP to bound ADP, be different from that purified under denaturing conditions.

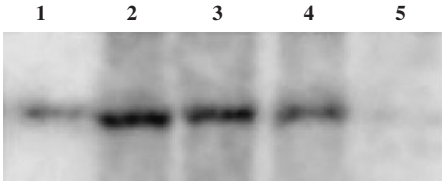

Fig. 1. **Western blot analysis of *M. tuberculosis* cell lysates.** *Mycobacterium tuberculosis* cell wall, membranes, whole-cell lysate and cytosolic fractions were obtained from 'Tuberculosis Research Materials' at Colorado State University, Forcollins, CO, under NIH contract. Approximately 5 μg of each fraction was resolved on SDS–PA gels, blotted and probed with antibody raised against anti-DnaA$_{TB}$ antibody. Lane 1, DnaA marker; lanes 2, whole-cell lysates; lane 3, membrane fraction; lane 4, soluble cell wall fraction; lane 5, cytosolic fraction.

ATPase Activity—We detected the ATPase activity of the nDnaA$_{TB}$ by PEI-thin layer chromatography. The DnaA protein showed a weak ATPase activity with turnover (catalytic-centre activity) of 7.5×10^{-4} (min^{-1}). Similar results were obtained with the denatured DnaA, although the denatured DnaA exhibited an ATPase activity with turnover number of 0.016 (min^{-1}) (*12*). It should be noted that the DnaA proteins of both *E. coli* and *Bacillus subtilis* also hydrolyse ATP slowly (*6*, *18*). The slow turnover rate suggests that either the ATPase activity of *M. tuberculosis* DnaA is intrinsically low and or that the protein retains ADP following the hydrolysis of ATP. Determination of radiolabelled nucleotide retained by DnaA as a function of time showed a slow time-dependent decrease in bound ATP with a concomitant increase in bound ADP (Fig. 3A). Hydrolysis of the tightly bound ATP in the presence of *oriC* was 50% complete in about 3 min, whereas in the absence of *oriC*, 50% hydrolysis was achieved in about 10 min (Fig. 3A), suggesting that *M. tuberculosis oriC* stimulated the ATPase activity of the native DnaA. In case of the denatured DnaA, hydrolysis of bound ATP was 50% complete in ~44 min without *oriC*, whereas it took 22 min in the presence of *oriC* (Fig. 3B).

Exchange of ATP for Bound ADP—The observed weak ATPase activity of DnaA$_{TB}$ suggested the exchange of ATP for bound ADP is very low. To test this hypothesis, DnaA protein–[^{14}C]ADP complexes were prepared first and then challenged with [α-^{32}P]ATP. After incubation for different periods, samples were removed, filtered and the amount of [α-^{32}P]ATP bound to DnaA was determined by scintillation counting. Parallel change experiments with non-radiolabelled ATP were carried out to

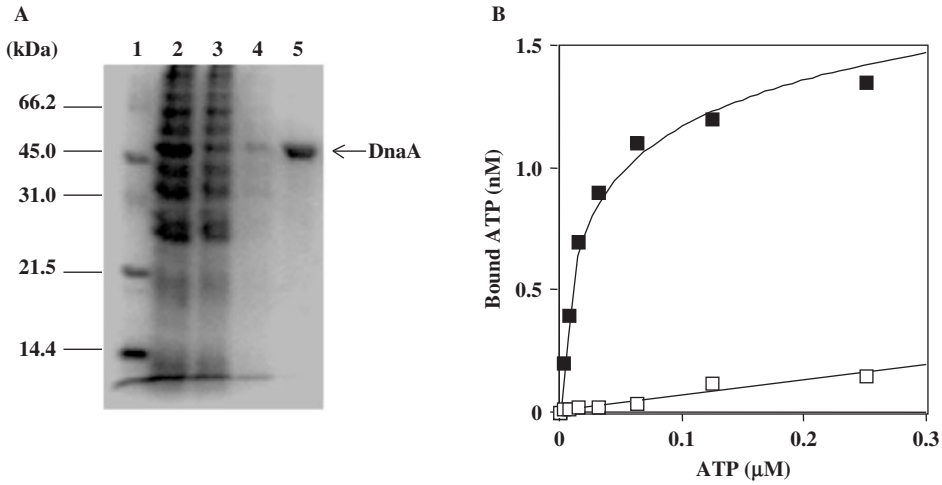

Fig. 2. **Purification and ATP-binding of *M. tuberculosis* DnaA protein.** (A) Protein fractions from the Ni-column were applied to an SDS–polyacrylamide gel with a 10% separating gel and stained with Coomassie brilliant blue R-250. Lane 1, molecular mass markers; lane 2, total lysate; lane 3, flow-through fraction; lane 4, washed fraction; lane 5, eluate (with 100 mM imidazole). (B) Each DnaA protein was incubated at 37°C for 15 min with indicated concentration of [α-^{32}P]ATP in buffer E. At the end of incubation, samples were collected on nitrocellulose filters and the radioactivity was measured as described in MATERIALS AND METHODS section. The amounts of ATP bound to DnaA$_{TB}$ (closed squares) and nDnaA$_{TB}$ (open squares) were plotted and K_D for ATP binding was determined.

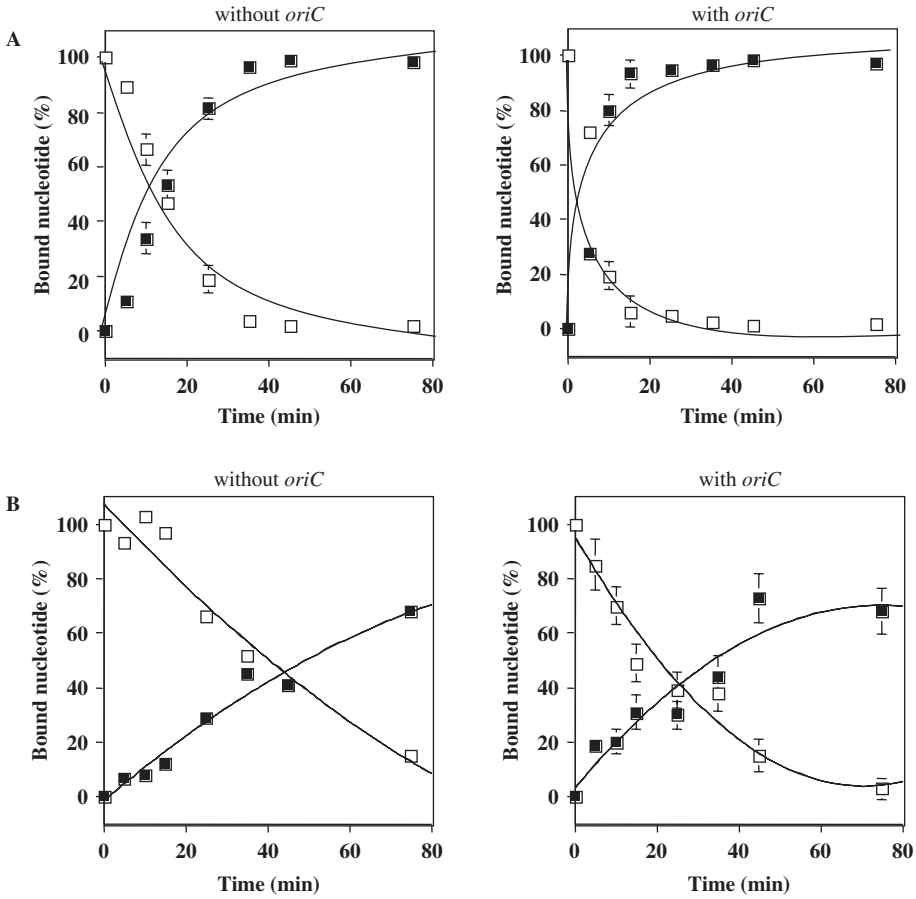

Fig. 3. **Effect of *oriC* on ATPase activity of the native DnaA (A) and the denatured DnaA (B).** Each DnaA protein (40 pmol) was incubated in buffer E (40 μl) at 0°C for 15 min with [α-^{32}P]ATP (100 pmol), and then at 37°C with or without *ori*C DNA. Samples were filtered on nitrocellulose membranes and extracted with 50 ml of 1 M HCOOH. Samples (0.5 μl) were spotted on thin layer PEI-cellulose plates. Chromatography was with 1 M HCOOH–0.4 M LiCl. Open and closed squares represent bound ATP and ADP, respectively. The relative intensities of ATP and ADP were quantified by densitometry scanning and the amount of ATP at time 0 was set to value of 100%.

determine the amount of ADP dissociated. It took ~10 min to exchange 50% of the bound ADP for ATP (Fig. 4). On the other hand, 70 min was needed for the exchange with the denatured DnaA (*12*). The observed decrease in ATP-binding after 20 min (Fig. 4) is due to the fact that release of bound nucleotide from DnaA protein was promoted when purified under native conditions. With the native DnaA, exchange of ATP for bound ADP was also facilitated.

Considering the presence of phospholipid phosphorous in the chloroform–methanol extracted samples of $nDnaA_{TB}$ (this study), and the ability of phospholipids to promote dissociation of bound nucleotides from DnaA purified under denaturing conditions (*12*), we reason that $DnaA_{TB}$ association with phospholipids could account for the observed differences in activities between the two protein preparations. It should be noted that solubilization of the DnaA with urea treatment was effective for the removal of any nucleotides and phospholipids associated with DnaA, much like the situation reported in *E. coli* (*19*). Phospholipids have been hypothesized to regulate DnaA activity of *E. coli oriC* replication (*20*). Presumably, this may well be the case with mycobacteria, although the nature of the individual phospholipids that regulate DnaA activity may be different in both organisms. One characteristic feature of mycobacteria is their lipid-rich cell walls (*21*, *22*). Phosphatidylinositol is one of the major phospholipids of *M. tuberculosis* and it has been shown to be the most effective agent for the dissociation of DnaA to ATP and ADP (*12*).

It is hypothesized that for the acidic phospholipid-mediated dissociation of adenine nucleotide bound to DnaA protein, electrostatic interactions followed by insertion of a part of the DnaA protein into the membrane is required (*23*). On the basis of two potential amphipathic helices in the *E. coli* DnaA protein (K327-I344 and D357-V374) responsible for interactions with acidic phospholipids (*24*, *25*), we found a corresponding membrane-binding domain in the *M. tuberculosis* DnaA protein that constitutes helix 1 (A393-D405) and sub-helices (D355-R367 and I369-A3810). Our results tend to suggest that the *M. tuberculosis* DnaA protein interacts with and possibly attaches to the cell membrane *in vivo*.

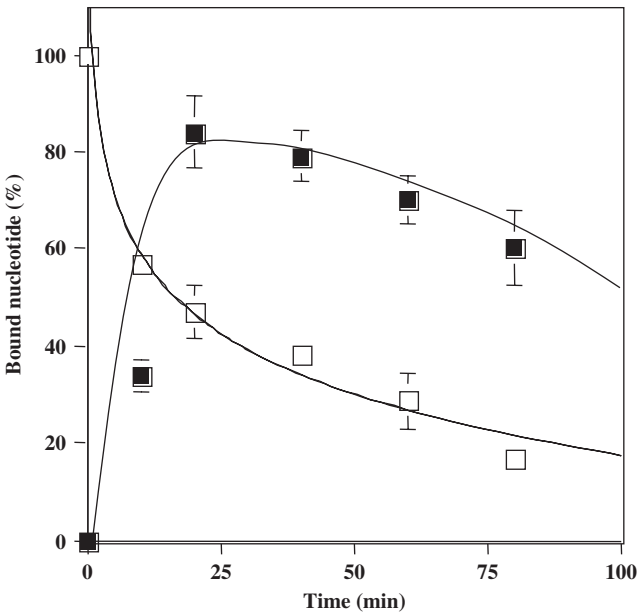

Fig. 4. **Exchange of ATP with ADP bound to DnaA protein.** $nDnaA_{TB}$ (2.0 pmol) was incubated with 200 pmol of [^{14}C]ADP in buffer E (100 μl) under standard condition, and then exposed to 1.0 mM [α-^{32}P]ATP. The samples were filtered on membranes at the indicated time periods. The amounts of ATP (closed squares) and ADP (open squares) bound to DnaA protein were plotted. Values of 100% correspond to the ADP retained by $nDnaA_{TB}$ protein at time 0.

Ichihashi and his colleagues (*23*) reported that the inhibitory effect of basic or neutral phospholipid on the interaction of acidic phospholipids with DnaA protein in *E. coli*. The biochemical findings suggest that DnaA activity is regulated by a change in the phospholipid composition of the cell membrane under physiological conditions. Although this may be an essential feature of the regulation of *M. tuberculosis oriC* replication, the nature of the rejuvenation of *M. tuberculosis* DnaA remains uncertain. Considering the complex nature of mycobacterial cell membranes, further studies are required to examine the effects of phospholipids on the regulation of DnaA activities

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