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RESEARCH ARTICLE

Association of ω with the C-Terminal Region of the β′ Subunit Is Essential for Assembly of RNA Polymerase in *Mycobacterium tuberculosis*

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ABSTRACT The ω subunit is the smallest subunit of bacterial RNA polymerase (RNAP). Although homologs of ω are essential in both eukaryotes and archaea, this subunit has been known to be dispensable for RNAP in *Escherichia coli* and in other bacteria. In this study, we characterized an indispensable role of the ω subunit in *Mycobacterium tuberculosis*. Unlike the well-studied *E. coli* RNAP, the *M. tuberculosis* RNAP core enzyme cannot be functionally assembled in the absence of the ω subunit. Importantly, substitution of *M. tuberculosis* ω with ω subunits from *E. coli* or *Thermus thermophilus* cannot restore the assembly of *M. tuberculosis* RNAP. Furthermore, by replacing different regions in *M. tuberculosis* ω with the corresponding regions from *E. coli* ω, we found a nonconserved loop region in *M. tuberculosis* ω essential for its function in RNAP assembly. From RNAP structures, we noticed that the location of the C-terminal region of the β′ subunit (β′CTD) in *M. tuberculosis* RNAP but not in *E. coli* or *T. thermophilus* RNAP is close to the ω loop region. Deletion of this β′CTD in *M. tuberculosis* RNAP destabilized the binding of *M. tuberculosis* ω on RNAP and compromised *M. tuberculosis* core assembly, suggesting that these two regions may function together to play a role in ω-dependent RNAP assembly in *M. tuberculosis*. Sequence alignment of the ω loop and the β′CTD regions suggests that the essential role of ω is probably restricted to mycobacteria. Together, our study characterized an essential role of *M. tuberculosis* ω and highlighted the importance of the ω loop region in *M. tuberculosis* RNAP assembly.

IMPORTANCE DNA-dependent RNA polymerase (RNAP), which consists of a multisubunit core enzyme ($\alpha_2\beta\beta'\omega$) and a dissociable σ subunit, is the only enzyme in charge of transcription in bacteria. As the smallest subunit, the roles of ω remain the least well studied. In *Escherichia coli* and some other bacteria, the ω subunit is known to be nonessential for RNAP. In this study, we revealed an essential role of the ω subunit for RNAP assembly in the human pathogen *Mycobacterium tuberculosis*, and a mycobacterium-specific ω loop that plays a role in this function was also characterized. Our study provides fresh insights for further characterizing the roles of bacterial ω subunit.

KEYWORDS *Mycobacterium tuberculosis*, transcription, RNA polymerase, omega subunit

DNA-dependent RNA polymerase (RNAP) carries out transcription in all organisms (1–3). In bacteria, RNAP is composed of a multisubunit core enzyme ($\alpha_2\beta\beta'\omega$), with an additional subunit (σ) used for promoter binding (4). Although the specific functions of each subunit have been extensively studied (5, 6), the roles of the smallest subunit (ω) remain the least well studied.

In *Escherichia coli*, ω has been observed to play a part in RNAP assembly by

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facilitating the binding of β' to $\alpha_2\beta$ to generate the RNAP core enzyme (5, 7, 8). Recent studies have showed that the stringent response alarmone molecule ppGpp directly binds to the RNAP β'-ω interface (9, 10) and regulates transcriptions from a subset of promoters (11, 12). However, deletion of ω in *E. coli* has no major impact on bacterial growth or RNAP activity (10, 13, 14), suggesting that ω is unnecessary for *E. coli* RNAP. Other studies have shown that ω is involved in either RNAP stability in *Staphylococcus aureus* (15) and or RNAP core-σ interaction in cyanobacteria (16), suggesting that the ω subunit may have diverse functions.

In *Mycobacterium tuberculosis*, the roles of the ω subunit have been poorly characterized. Multiple lines of evidence have indicated that ω plays an important role in mycobacteria. First, *Mycobacterium leprae*, which has lost many genes during the process of reductive evolution, still preserves the ω subunit (8, 17). Second, deletion of *rpoZ*, a gene encodes the ω subunit, significantly decreased the growth rate of *Mycobacterium smegmatis* and resulted in proteolytic cleavage of the RNAP β' subunit (18). Third, *rpoZ* has been predicted to be an essential gene in *M. tuberculosis* by transposon mutagenesis screening (19, 20). However, how the ω subunit functions in RNAP in mycobacteria is not fully understood.

In this study, we investigated the roles of ω in *M. tuberculosis* RNAP. In contrast to *E. coli* RNAP, ω is critical for the assembly of *M. tuberculosis* RNAP. Using a combination of sequence alignment, structural comparison, and mutational analyses, we demonstrated that a loop region of the ω subunit plays an essential role in the ω-dependent *M. tuberculosis* RNAP assembly. These findings highlight the importance of ω in *M. tuberculosis* and offer fresh insights into the roles of the bacterial ω subunit.

RESULTS

The ω subunit is required for *M. tuberculosis* RNAP assembly. To examine the role of the *M. tuberculosis* ω subunit, we first purified *M. tuberculosis* RNAP core (abbreviated as *M. tuberculosis* core, containing a His_6 tag at the C terminal of the β' subunit) with or without ω using coexpression plasmids (pMtRc and pMtRc$\Delta\omega$, respectively). As shown in Fig. 1A, a significant loss of α and β subunits from *M. tuberculosis* core was observed when ω was absent. Coexpression of *M. tuberculosis* ω^{Flag} protein with *M. tuberculosis* core$\Delta\omega$ in *E. coli* successfully restored RNAP assembly (Fig. 1A). In contrast, assembly of *E. coli* RNAP core (abbreviated as *E. coli* core) did not rely on ω (Fig. 1B). Furthermore, *in vitro* RNAP reconstitution using individual subunits (α^{His}, β, β', and ω^{His}) also showed that, in contrast to *E. coli* core, the *M. tuberculosis* core required the ω subunit for assembly (Fig. 1C).

In vitro abortive transcription at the *Rv1494* promoter (*Rv1494*p) (see Fig. S1A in the supplemental material) showed that *M. tuberculosis* core$\Delta\omega$, when purified from *in vivo* assembly or *in vitro* reconstitution, was inactive when reconstituted with the principle factor σ^A (Fig. 1D and E), which was also confirmed by transcription assays using a different sigma factor (σ^B) (see Fig. S1B in the supplemental material) or at a different promoter (*Rv0005*p) (see Fig. S1C in the supplemental material). These data suggest that the defect in *M. tuberculosis* core$\Delta\omega$ assembly influenced the activity of RNAP core enzyme. To test this hypothesis, we applied a σ-independent transcription elongation assay (21) to compare the activities of *M. tuberculosis* core with or without ω and found that *M. tuberculosis* core$\Delta\omega$ was inactive in catalysis of RNA synthase (see Fig. S1D and S1E in the supplemental material). In contrast, *E. coli* core$\Delta\omega$ from *in vivo* or *in vitro* assembly was active and showed more resistance to ppGpp than wild-type *E. coli* core (10, 22) (see Fig. S1F and S1G in the supplemental material). The loss in the function of *M. tuberculosis* core$\Delta\omega$ did not result from the purification procedures, since *M. tuberculosis* core$\Delta\omega$ was inactive after the first step of purification (see Fig. S1H in the supplemental material). Collectively, these data suggest that ω plays a more important role in assembling a functional RNAP in *M. tuberculosis* compared to that in *E. coli*.

The ω loop is essential for *M. tuberculosis* RNAP assembly. To explore the reason for the different actions of *M. tuberculosis* ω in RNAP assembly from that of *E. coli* ω, we aligned the amino acid sequences of ω from *M. tuberculosis*, *E. coli*, and *T. thermophilus*

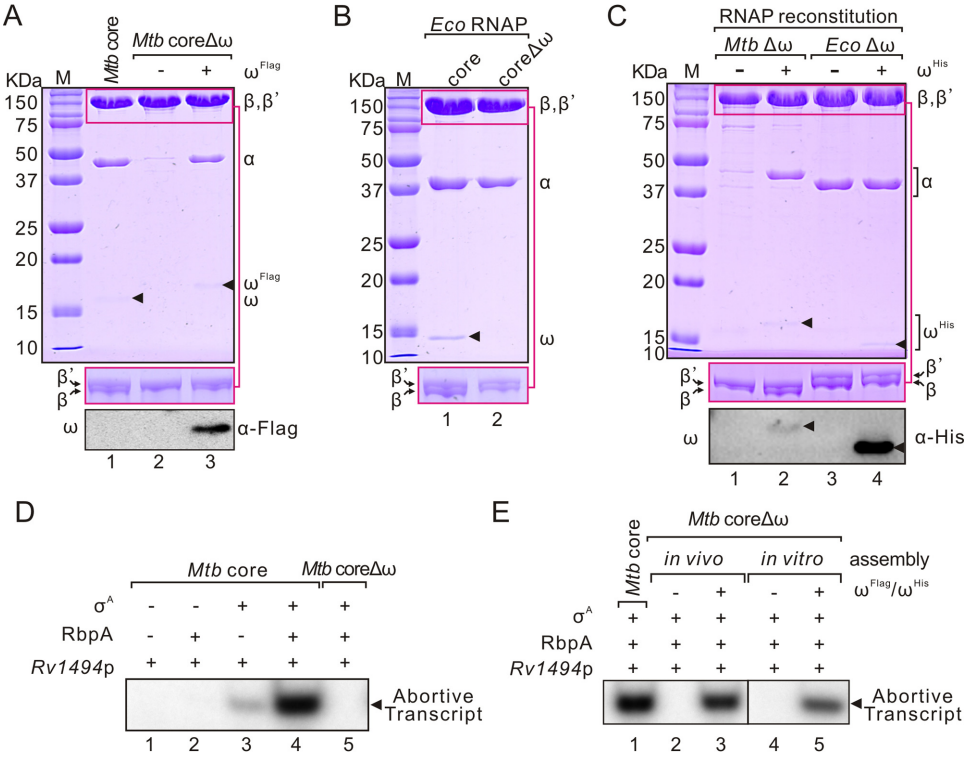

FIG 1 Roles of ω in *M. tuberculosis* and *E. coli* RNAP core assembly. (A) SDS-PAGE analysis of purified *M. tuberculosis* RNAP core, coreΔω, and coreΔω coexpressed with ω^{Flag} protein. Proteins loaded onto 4 to 20% gel to separate β and β′ subunits are shown in the middle panel. Western blots of ω^{Flag} with a Flag tag antibody are shown in the bottom panel. (B) Purified *E. coli* core and coreΔω. (C) *In vitro* reconstitution of *M. tuberculosis* and *E. coli* core with or without ω^{His}. Western blots of ω^{His} with a His tag antibody are shown in the bottom panel. (D) *In vitro* abortive transcription of purified *M. tuberculosis* core or coreΔω at the *Rv1494* promoter (*Rv1494*p). RbpA and σ^A were added as indicated to reconstitute RNAP for promoter-dependent transcription. (E) *In vitro* abortive transcriptional activities of *in vivo* or *in vitro* reconstituted *M. tuberculosis* core in the presence or absence of the ω subunit at *Rv1494*p. RbpA and σ^A were added in each reaction in promoter-dependent transcription assay. A representative result from three independent tests is shown. *Mtb*, *M. tuberculosis*; *Eco*, *E. coli*.

and found that the sequences of ω subunits were not conserved among these strains (Fig. 2A). Therefore, we tried to coexpress the ω from *E. coli* or *T. thermophilus* with *M. tuberculosis* coreΔω to test whether *E. coli* or *T. thermophilus* ω can complement the assembly of *M. tuberculosis* coreΔω. As shown in Fig. 2B, neither *E. coli* nor *T. thermophilus* ω rescued *M. tuberculosis* coreΔω assembly, although *T. thermophilus* ω was able to bind to *M. tuberculosis* RNAP. Based on the sequence conservation, we divided ω into three regions: the nonconserved N-terminal R1, the relatively conserved R2, and the nonconserved C-terminal R3 (Fig. 2A). To identify the specific region that is essential for the function of *M. tuberculosis* ω, we constructed and expressed three *M. tuberculosis* ω mutants ($\omega^{E\text{-}R1}$, $\omega^{E\text{-}R2}$, and $\omega^{E\text{-}R3}$) by replacing these three regions with the corresponding regions from *E. coli* ω. Western blot analysis showed that these mutant proteins were all solubly expressed (see Fig. S2A in the supplemental material). Interestingly, replacement of the relatively conserved R2 region but not the R1 or R3 region disrupted the binding of ω on RNAP and the function of *M. tuberculosis* core (Fig. 2C and D).

Structural superposition of the ω R2 regions from *M. tuberculosis*, *E. coli*, and *T. thermophilus* (23–25) showed that the loop regions differ greatly in both sequence and conformation (Fig. 3A and B), indicating that this loop region may be responsible for the different actions of ω. To test this hypothesis, we replaced the loop region of *M. tuberculosis* ω (amino acids [aa] 65 to 89) with the corresponding regions from *E. coli* (aa 31 to 44) or *T. thermophilus* (aa 31 to 58) to generate two mutants named as $\omega^{E\text{-}loop}$ and $\omega^{T\text{-}loop}$. Although these proteins were all solubly expressed (see Fig. S2B in the supplemental material), complementation of $\omega^{E\text{-}loop}$ or $\omega^{T\text{-}loop}$ mutant affected the

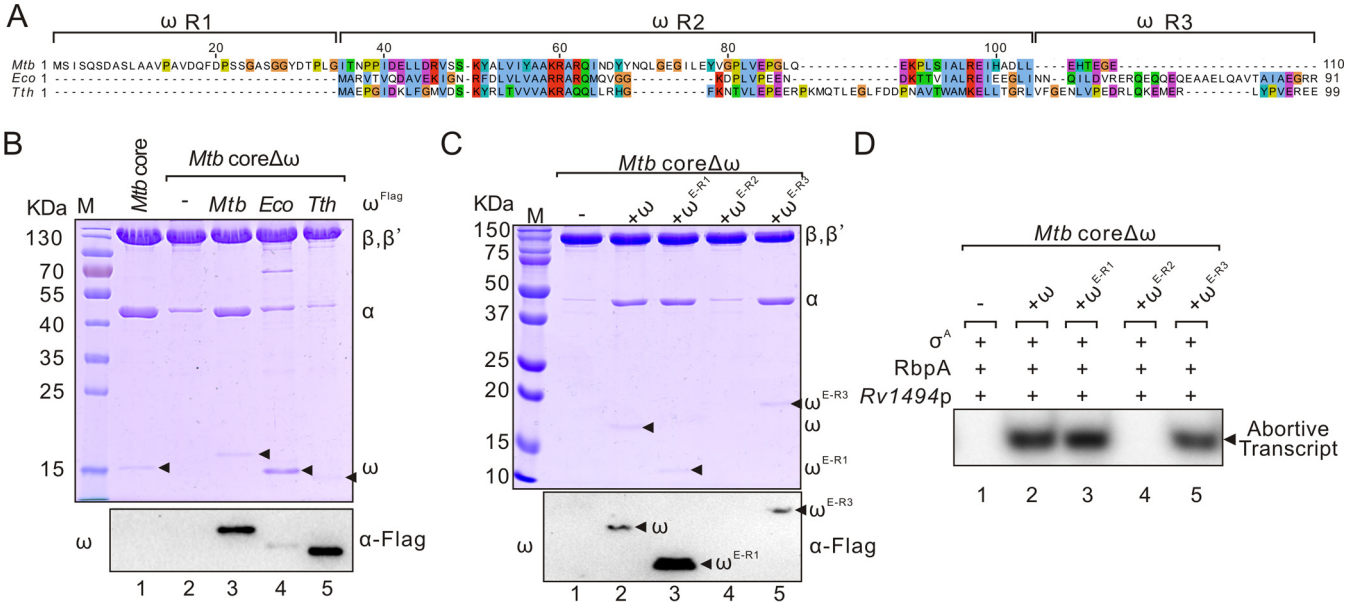

FIG 2 The relatively conserved R2 region is essential for the function of *M. tuberculosis* ω. (A) Sequence alignment of the ω subunits from *M. tuberculosis*, *E. coli*, and *T. thermophilus*. The amino acid numbering shown on top of the this figure is for *M. tuberculosis* ω. (B) SDS-PAGE analysis of purified *M. tuberculosis* coreΔω coexpressed with Flag-tagged ω from *M. tuberculosis*, *E. coli*, or *T. thermophilus*. Western blots of ω^{Flag} with a Flag tag antibody are shown in the bottom panel. (C) Purified *M. tuberculosis* core with a mutated ω subunit. *M. tuberculosis* ω mutants, in which the R1, R2, or R3 regions of *M. tuberculosis* ω was replaced with the corresponding regions of *E. coli* ω ($\omega^{E\text{-}R1}$, $\omega^{E\text{-}R2}$, and $\omega^{E\text{-}R3}$), respectively, were coexpressed with *M. tuberculosis* coreΔω in *E. coli*. Western blots of mutated ω^{Flag} subunit with a Flag tag antibody are shown in the bottom panel. (D) *In vitro* abortive transcription of purified *M. tuberculosis* coreΔω derivatives at *Rv1494*p. RbpA and σ^A were added in each reaction in this promoter-dependent transcription assay. A representative result from three independent tests is shown. *Mtb*, *M. tuberculosis*; *Eco*, *E. coli*; *Tth*, *T. thermophilus*.

assembly and function of *M. tuberculosis* coreΔω, despite the fact that $\omega^{T\text{-}loop}$ can bind to *M. tuberculosis* RNAP (Fig. 3C). Replacement of amino acids in the *M. tuberculosis* ω loop region with a flexible sequence (GSGGS) further proved that this region, especially N_{65}-N_{69} amino acids, is important for RNAP assembly (see Fig. S3 in the supplemental

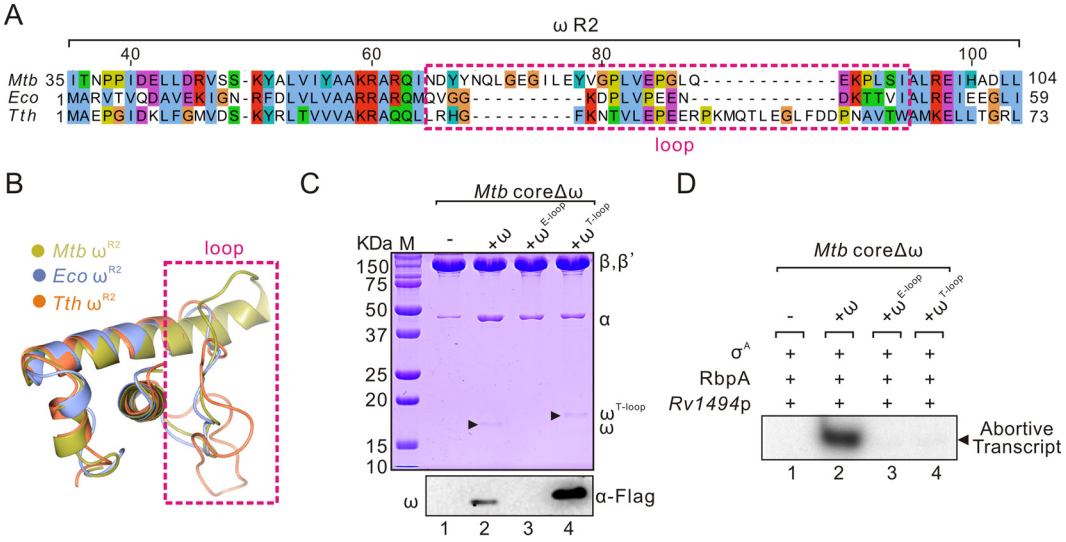

FIG 3 The ω loop is required for the assembly of *M. tuberculosis* core. (A) Sequence alignment of the R2 region in ω subunits from *M. tuberculosis*, *E. coli*, and *T. thermophilus*. The ω loop region is marked with a dashed box. The amino acid numbering in top part is for *M. tuberculosis* ω. (B) Structural overlay of the ω R2 regions in *M. tuberculosis* (aa 35 to 104, yellow, PDB 5UH8), *E. coli* (aa 1 to 59, blue, PDB 4YG2), and *T. thermophilus* (aa 1 to 73, orange, PD 1IW7). (C) Purified *M. tuberculosis* core with a mutated ω subunit carrying the loop regions from *E. coli* (aa 31 to 44) or *T. thermophilus* (aa 31 to 58) ($\omega^{E\text{-}loop}$ and $\omega^{T\text{-}loop}$, respectively). (D) *In vitro* abortive transcription of purified *M. tuberculosis* coreΔω derivatives at *Rv1494*p. RbpA and σ^A were added in each reaction. *Mtb*, *M. tuberculosis*; *Eco*, *E. coli*; *Tth*, *T. thermophilus*.

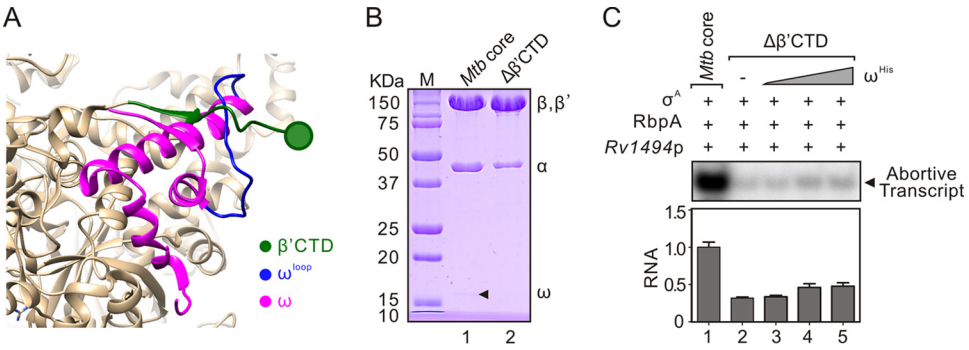

FIG 4 The β′CTD stabilizes the ω in *M. tuberculosis* core. (A) Structure of *M. tuberculosis* RNAP core with the magnified view showing the β′ C-terminal domain (aa 1271 to 1316, β′ CTD, green), ω subunit (magenta), and ω loop region (aa 65 to 89, blue) (PDB 5UH8). (B) SDS-PAGE of the purified *M. tuberculosis* core and coreΔβ′CTD. (C) *In vitro* abortive transcription of purified *M. tuberculosis* core or coreΔβ′CTD at *Rv1494*p. Activities of *M. tuberculosis* coreΔβ′CTD (200 nM) with addition of purified ω^{His} (200, 400, or 800 nM, respectively) were tested. RbpA and σ^A were added in each reaction. A representative transcription result from two independent tests is shown in each panel. Quantifications of transcripts from two independent tests are shown in each bottom section. *Mtb*, *M. tuberculosis*.

material). These data provide further evidence that the loop region is essential for the role of *M. tuberculosis* ω.

The β′CTD plays a role in ω-dependent RNAP assembly. From the structure of *M. tuberculosis* RNAP (23, 26), we noticed that the ω loop locates on the outside surface of RNAP and probably associates with the C-terminal region of the *M. tuberculosis* β′ subunit (aa 1271 to 1316, β′CTD) (Fig. 4A). Therefore, we tested the importance of this observed interaction in the assembly of *M. tuberculosis* RNAP. For this purpose, we constructed a plasmid expressing *M. tuberculosis* core without the β′CTD region (pMtRcΔβ′CTD). Compared to wild-type *M. tuberculosis* core, the amounts of ω and α subunits were decreased in purified *M. tuberculosis* coreΔβ′CTD (Fig. 4B; also see Fig. S4 in the supplemental material), suggesting that the β′CTD is important for ω stability and core assembly in *M. tuberculosis* RNAP. *In vitro* abortive initiation assays also showed that the *M. tuberculosis* coreΔβ′CTD was defective in transcription compared to *M. tuberculosis* core (Fig. 4C). Interestingly, the addition of purified ω subunit to *M. tuberculosis* coreΔβ′CTD (ω:coreΔβ′CTD, 4:1) only slightly restored its transcription activity (Fig. 4C), suggesting that the β′CTD is essential for the function of the ω subunit in *M. tuberculosis* core. Based on the facts that the β′CTD and ω loop regions are located closely in *M. tuberculosis* RNAP structure and *M. tuberculosis* core with mutation of either of these two regions behaved similarly in assembly and function, we suggest that the *M. tuberculosis* β′CTD may associate with the ω loop region to stabilize the ω subunit in *M. tuberculosis* RNAP and facilitate the ω-dependent RNAP assembly.

The role of ω is probably conserved in mycobacteria. To test whether the role of ω subunit, especially the association between the ω loop and β′CTD, in RNAP assembly is also essential in other bacteria, we aligned the protein sequences of the ω and β′ subunits from different bacteria. Amino acid sequences of the ω loop and the β′CTD regions are highly conserved in mycobacteria and moderately conserved in actinobacteria (Fig. 5A). We therefore expressed and purified the RNAP core and coreΔω from *M. smegmatis*, *Streptomyces coelicolor*, *Pseudomonas aeruginosa*, and *T. thermophilus*. As expected, the absence of the ω subunit had a marked impact on the assembly and function of *M. smegmatis* core as observed for *M. tuberculosis* core but not those from *P. aeruginosa*, *T. thermophilus*, or *E. coli* (Fig. 5B and C). Consistently, the location of β′CTD is close to the ω loop in *M. smegmatis* RNAP structure (27) but not in *E. coli* (25) and *T. thermophilus* (24) RNAPs (see Fig. S5 in the supplemental material). Interestingly, although the *S. coelicolor* and mycobacterial core sequences are similar, ω is not required for the assembly and function of *S. coelicolor* RNAP (Fig. 5B and C). Complementation of *M. smegmatis* coreΔω with ω^{Flag} fully restored its assembly and function

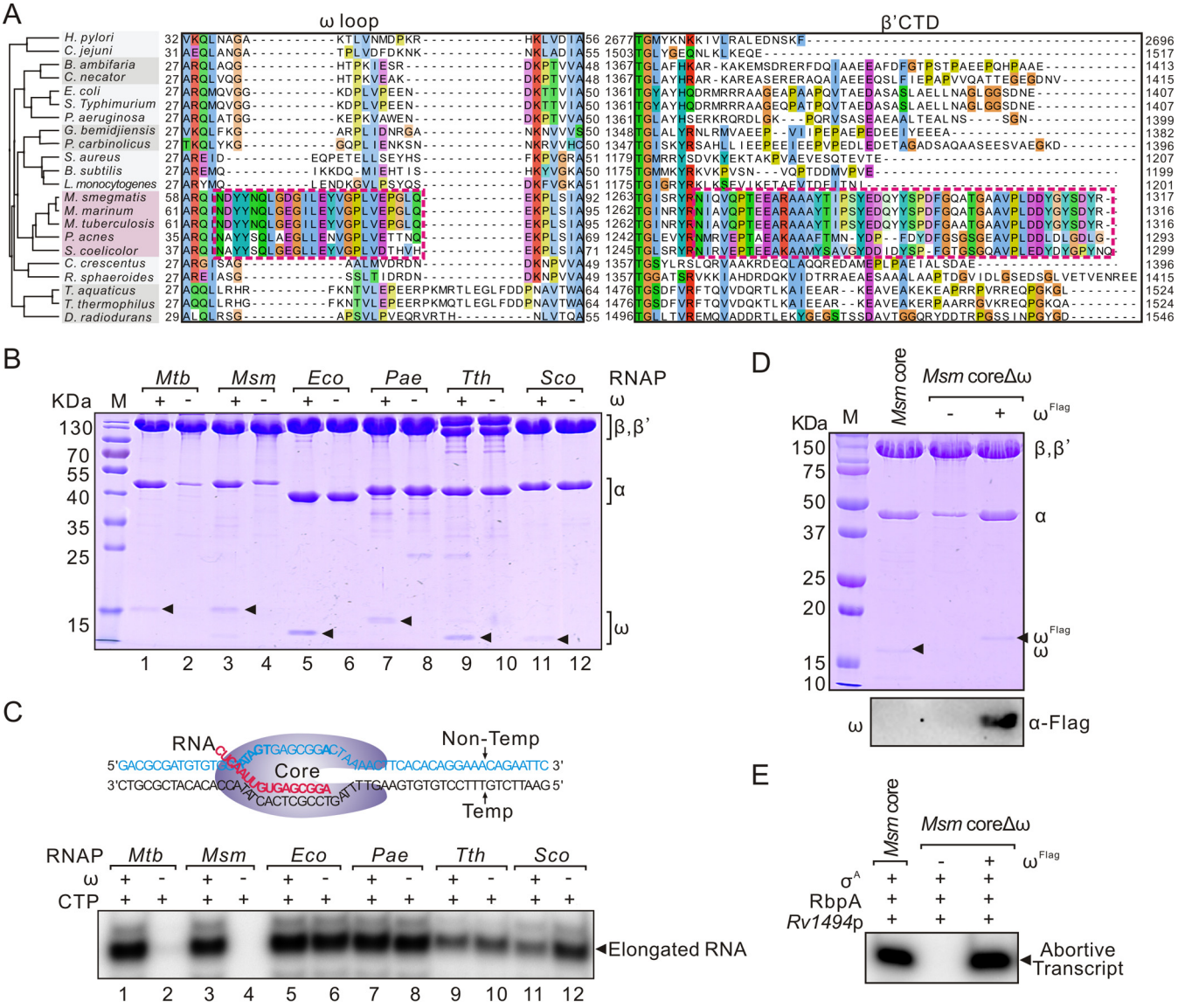

FIG 5 Roles of ω are conserved in mycobacteria. (A) Sequence alignments of the ω loop and β'CTD regions from different bacteria. A phylogenetic tree constructed with bacterial 16S rRNA genes is shown in the left section. Bacterial species used: *Helicobacter pylori*, *Campylobacter jejuni*, *Burkholderia ambifaria*, *Cupriavidus necator*, *E. coli*, *Salmonella enterica* serovar Typhimurium, *P. aeruginosa*, *Geobacter bemidjiensis*, *Pelobacter carbinolicus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes*, *M. smegmatis*, *M. marinum*, *M. tuberculosis*, *Propionibacterium acnes*, *S. coelicolor*, *Caulobacter crescentus*, *Rhodobacter sphaeroides*, *Thermus aquaticus*, *T. thermophilus*, and *Deinococcus radiodurans*. Regions conserved in actinobacteria are shown in a dashed box. (B) Purified *M. tuberculosis*, *M. smegmatis*, *E. coli*, *P. aeruginosa*, *T. thermophilus*, and *S. coelicolor* RNAP cores with or without the ω subunits. The ω subunits are marked with black triangles. (C) Transcriptional activities of these core enzymes in a σ-independent transcription elongation assay. A scheme for this test is shown at the upper part. The RNA primer is shown in red. [α-^{32}P]CTP was added as the substrate for elongating RNA primer. (D) Complementation of *M. smegmatis* core$\Delta\omega$ by coexpression with *M. smegmatis* ω^{Flag}. Assemblies of these RNAPs are shown in the upper section, and Western blotting of ω^{Flag} with a Flag tag antibody is shown in the bottom panel. (E) *In vitro* abortive transcription of *M. smegmatis* core and core$\Delta\omega$ at *Rv1494*p. RbpA and σ^A were added in each reaction to reconstitute RNAP for promoter-dependent transcription.

(Fig. 5D and E). Collectively, these results demonstrate that the essential role of ω in RNAP assembly is probably conserved in mycobacteria.

DISCUSSION

As an RNAP subunit, ω and its homologs are present in all sequenced genomes of free-living organisms. Although its homologs (RpoK and RPB6) are essential in archaea and eukaryotes (28), ω has been shown to be nonessential in several bacteria (29–32). In *M. tuberculosis*, the roles of ω have not been clearly investigated (33–35). In this study, we showed that the ω subunit is essential for the assembly of *M. tuberculosis*

RNAP core and have characterized a loop region in *M. tuberculosis* ω which is essential for its roles.

The roles of ω are diverse in bacteria. Although ω has been observed to play a part in RNAP core assembly in *E. coli* (7, 13), *E. coli* core could assemble well without ω, both *in vitro* and *in vivo* (13, 36) (Fig. 1), suggesting that the role of the ω subunit in *E. coli* RNAP assembly is nonessential. In this study, we provide clear evidence (*in vivo* and *in vitro* assembly) that, in contrast to *E. coli*, *M. tuberculosis* core could not be well assembled and almost lost its function in the absence of ω. In addition, a previous study has shown that deletion of the *rpoZ* gene in *M. smegmatis* resulted in proteolytic cleavage of RNAP β′ subunit and further affected the function of RNAP *in vivo* (18). All these data indicate that mycobacterial ω plays more important roles than that of *E. coli* ω. A loss of α and β subunits in RNAP was observed in the absence of the ω subunit when His-tagged β′ was used in purification, indicating that ω might facilitate the binding of $\alpha_2\beta$ to β′, as was reported in *E. coli* (7). Regardless, the detailed contributions of ω to RNAP assembly in *M. tuberculosis* and *E. coli* may be different and require further studies.

To the best of our knowledge, the role of the loop region in the middle part of ω subunit in RNAP assembly has not been reported. In this study, we showed that mutation of this loop region in *M. tuberculosis* ω compromised the assembly of *M. tuberculosis* core and the structurally closely located C-terminal region of the β′ subunit is also involved in the roles of ω subunit in *M. tuberculosis* core. Although structures of both *M. tuberculosis* and *M. smegmatis* RNAP have been determined, a fraction of the β′CTD has not been fully resolved in either model (23, 26, 27, 37). Anyhow, this β′CTD region is close to the ω loop region in both *M. tuberculosis* and *M. smegmatis* RNAP structures but not in the *E. coli* and *T. thermophilus* RNAP structures (24, 25, 38), suggesting that this species-specific association between the ω loop and the β′CTD regions may contribute to the essential role of ω subunit in RNAP assembly in mycobacteria.

Our sequence alignment showed that the β′CTD and ω loop regions, which determine the dependence of *M. tuberculosis* RNAP for ω, are conserved in mycobacteria. Although the sequences of these two regions from *S. coelicolor*, a representative species of actinobacteria, are similar to those from mycobacteria, our data showed that the role of ω in *S. coelicolor* is not essential, which is in consistent with a previous study (30) but is different from that of mycobacteria. These differences indicate that amino acids that are distinct between mycobacteria and *S. coelicolor* in these conserved regions or some additional element(s) may be required to determine the specific features of RNAP.

Although we have shown a strict dependence for ω by the mycobacterial RNAP core *in vitro*, deletion of a region in the *rpoZ* gene has been successfully constructed in *M. smegmatis*, although the growth of this strain is strongly repressed (18). Whether the role of ω could be complemented in part by other factors in mycobacteria, such as homologs of GroEL in *E. coli* (32), is worthy of further investigation. The lack of a proper chaperone(s) may partly explain the defects in RNAP assembly observed in *M. tuberculosis* coreΔω. However, *E. coli* coreΔω, but not the *M. tuberculosis* coreΔω, is well assembled in *in vitro* reconstitution experiments, which suggests that the role of ω subunit is more important in *M. tuberculosis* than in *E. coli*. Nevertheless, our study provides fresh insights for further characterizing the roles of the bacterial ω subunit in transcription.

MATERIALS AND METHODS

Plasmids, oligonucleotides, and bacterial growth conditions. The plasmids used in this study are summarized in Table S1 in the supplemental material. The oligonucleotides used in plasmid constructions are listed in Table S2 in the supplemental material. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar-solidified plates at 37°C or at other temperatures, as indicated.

Protein expression and purification. Plasmids expressing RNAPs or associated proteins were constructed using a ClonExpress II one-step cloning kit (Vazyme, China). Mutations in genes were constructed with a QuikChange II XL site-directed mutagenesis kit (Stratagene). Bacterial RNAPs (with a His tag at the C terminal of β′ subunit) expressed in *E. coli* BL21(DE3) were purified as we previously described (21). For a detailed description of the methods, see the supplemental material.

RNAP reconstitution. RNAP reconstitution was performed as previously described (36, 39). Briefly, *M. tuberculosis* and *E. coli* α^{His} and ω^{His} subunits were expressed in *E. coli* BL21(DE3) and subsequently purified with Ni-resin. The *M. tuberculosis* and *E. coli* β and β' subunits were overexpressed in *E. coli* BL21(DE3) and isolated from inclusion bodies. The RNAP subunits were mixed at a molar ratio of 1:1.5:3:3 for $\beta':\beta:\alpha:\omega$ (when ω was present) in 10 ml of denaturing buffer (50 mM Tris-HCl [pH 7.9], 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM EDTA, 10 mM dithiothreitol [DTT], 10% glycerol, 6 M guanidine hydrochloride). The mixtures were then dialyzed against refolding buffer (50 mM Tris-HCl [pH 7.9], 200 mM KCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM EDTA, 5 mM 2-mercaptoethanol, 20% glycerol) at 4°C for 16 h, with two rounds of buffer changes. Next, the reconstitution mixtures were incubated at 30°C for 60 min and centrifuged at 13,000 $\times$ *g* for 10 min at 4°C. RNAPs were then purified with heparin and MonoQ columns. Details regarding the protein purification methods are provided in the supplemental material.

Western blot analysis. Samples were resolved by SDS–12% PAGE and transferred onto polyvinylidene difluoride membranes. After being blocked with 5% (wt/vol) nonfat milk powder in phosphate buffer with 0.05% Tween 20 (PBST), the membranes were incubated with anti-Flag (mouse monoclonal; Sigma-Aldrich) or anti-His (mouse monoclonal; Beyotime Biotechnology) primary antibody (diluted 1:1,000). The membranes were washed three times with PBST and then incubated with a 1:10,000 dilution of a horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich) for 1 h. Signals were detected using an ECL kit (Bio-Rad) according to the manufacturer's protocol.

***In vitro* transcription.** For abortive initiation assay, reactions were performed as previously described (21, 40). Briefly, transcription was performed in 5 μl of transcription buffer (20 mM Tris-HCl [pH 7.9], 50 mM NaCl, 5 mM $MgSO_4$, 1 mM DTT, 0.1 mM EDTA, 5% glycerol). RNAP holoenzyme was assembled by mixing 200 nM RNAP core and 400 nM σ factor. The activator protein RbpA (21) was added at 800 nM for mycobacterial RNAP in transcription assays. A promoter fragment (30 nM) was incubated with RNAP at 37°C for 10 min. Transcription was initiated by the addition of 50 μM limited nucleoside triphosphates (GTP and UTP for *Rv1494*p and *rrn*P3; GTP and ATP for *Rv0005*p), together with 1 μCi of [α-^{32}P]CTP. The reactions were carried out at 37°C for 20 min for mycobacterial RNAP and for 5 min for *E. coli* RNAP.

The σ-independent transcription elongation reactions were performed as described previously (21). Briefly, 80 nM RNA (5′-CUCAAUUGUGAGCGGA-3′) and 40 nM template strand DNA (5′-GAATTCTGTTTC CTGTGTGAAGTTTTAGTCCGCTCACTATACCACACATCGCGTC-3′) were first annealed and then incubated with 200 nM RNAP core at 24°C for 30 min. Next, 40 nM nontemplate DNA (5′-GACGCGATGTGTGGTAT AGTGAGCGGACTAAAACTTCACACAGGAAACAGAATTC-3′) was added, followed by incubation at 37°C for 10 min. Transcription reactions were initiated by the addition of 1 μCi of [α-^{32}P]CTP or a mixture of 50 μM ATP, UTP, and GTP and 1 μCi of [α-^{32}P]CTP. Reactions were carried out at 24°C (for *M. tuberculosis*, *M. smegmatis*, *S. coelicolor*, *E. coli*, and *P. aeruginosa* RNAP) or 65°C (for *T. thermophilus* RNAP) for 5 min. Transcripts were analyzed on 20% denaturing PAGE (7 M urea) and detected by a cyclone storage phosphor system (Perkin-Elmer). All tests were performed at least twice independently. ImageJ software was used to quantify the products.

Statistical analysis. The experiments were analyzed using Student *t* tests and repeated three times. Statistical analyses were performed using GraphPad Prism software. Quantitative data are presented as mean values with standard deviations.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00159-18.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.

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REFERENCES

1. Werner F. 2008. Structural evolution of multisubunit RNA polymerases. Trends Microbiol 16:247–250. https://doi.org/10.1016/j.tim.2008.03.008.
2. Werner F, Grohmann D. 2011. Evolution of multisubunit RNA polymerases in the three domains of life. Nat Rev Microbiol 9:85–98. https://doi.org/10.1038/nrmicro2507.
3. Cramer P. 2002. Multisubunit RNA polymerases. Curr Opin Struct Biol 12:89–97. https://doi.org/10.1016/S0959-440X(02)00294-4.

4. Murakami KS, Darst SA. 2003. Bacterial RNA polymerases: the wholo story. Curr Opin Struct Biol 13:31–39. https://doi.org/10.1016/S0959-440X(02)00005-2.
5. Murakami KS. 2015. Structural biology of bacterial RNA polymerase. Biomolecules 5:848–864. https://doi.org/10.3390/biom5020848.
6. Borukhov S, Nudler E. 2003. RNA polymerase holoenzyme: structure, function and biological implications. Curr Opin Microbiol 6:93–100. https://doi.org/10.1016/S1369-5274(03)00036-5.
7. Ghosh P, Ishihama A, Chatterji D. 2001. *Escherichia coli* RNA polymerase subunit omega and its N-terminal domain bind full-length β′ to facilitate incorporation into the α2β subassembly. Eur J Biochem 268:4621–4627. https://doi.org/10.1046/j.1432-1327.2001.02381.x.
8. Mathew R, Chatterji D. 2006. The evolving story of the omega subunit of bacterial RNA polymerase. Trends Microbiol 14:450–455. https://doi.org/10.1016/j.tim.2006.08.002.
9. Zuo Y, Wang Y, Steitz TA. 2013. The mechanism of *E. coli* RNA polymerase regulation by ppGpp is suggested by the structure of their complex. Mol Cell 50:430–436. https://doi.org/10.1016/j.molcel.2013.03.020.
10. Ross W, Vrentas CE, Sanchez-Vazquez P, Gaal T, Gourse RL. 2013. The magic spot: a ppGpp binding site on *Escherichia coli* RNA polymerase responsible for regulation of transcription initiation. Mol Cell 50:420–429. https://doi.org/10.1016/j.molcel.2013.03.021.
11. Magnusson LU, Farewell A, Nystrom T. 2005. ppGpp: a global regulator in *Escherichia coli*. Trends Microbiol 13:236–242. https://doi.org/10.1016/j.tim.2005.03.008.
12. Maisonneuve E, Gerdes K. 2014. Molecular mechanisms underlying bacterial persisters. Cell 157:539–548. https://doi.org/10.1016/j.cell.2014.02.050.
13. Mukherjee K, Chatterji D. 1997. Studies on the omega subunit of *Escherichia coli* RNA polymerase: its role in the recovery of denatured enzyme activity. Eur J Biochem 247:884–889. https://doi.org/10.1111/j.1432-1033.1997.00884.x.
14. Sarkar P, Sardesai AA, Murakami KS, Chatterji D. 2013. Inactivation of the bacterial RNA polymerase due to acquisition of secondary structure by the omega subunit. J Biol Chem 288:25076–25087. https://doi.org/10.1074/jbc.M113.468520.
15. Weiss A, Moore BD, Tremblay MH, Chaput D, Kremer A, Shaw LN. 2017. The omega subunit governs RNA polymerase stability and transcriptional specificity in *Staphylococcus aureus*. J Bacteriol 199:e00459-16. https://doi.org/10.1128/JB.00459-16.
16. Gunnelius L, Hakkila K, Kurkela J, Wada H, Tyystjarvi E, Tyystjarvi T. 2014. The omega subunit of the RNA polymerase core directs transcription efficiency in cyanobacteria. Nucleic Acids Res 42:4606–4614. https://doi.org/10.1093/nar/gku084.
17. Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, Honore N, Garnier T, Churcher C, Harris D, Mungall K, Basham D, Brown D, Chillingworth T, Connor R, Davies RM, Devlin K, Duthoy S, Feltwell T, Fraser A, Hamlin N, Holroyd S, Hornsby T, Jagels K, Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail MA, Rajandream MA, Rutherford KM, Rutter S, Seeger K, Simon S, Simmonds M, Skelton J, Squares R, Squares S, Stevens K, Taylor K, Whitehead S, Woodward JR, Barrell BG. 2001. Massive gene decay in the leprosy bacillus. Nature 409:1007–1011. https://doi.org/10.1038/35059006.
18. Mathew R, Ramakanth M, Chatterji D. 2005. Deletion of the gene *rpoZ*, encoding the omega subunit of RNA polymerase, in *Mycobacterium smegmatis* results in fragmentation of the β′ subunit in the enzyme assembly. J Bacteriol 187:6565–6570. https://doi.org/10.1128/JB.187.18.6565-6570.2005.
19. Sassetti CM, Boyd DH, Rubin EJ. 2003. Genes required for mycobacterial growth defined by high-density mutagenesis. Mol Microbiol 48:77–84. https://doi.org/10.1046/j.1365-2958.2003.03425.x.
20. Griffin JE, Gawronski JD, Dejesus MA, Ioerger TR, Akerley BJ, Sassetti CM. 2011. High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. PLoS Pathog 7:e1002251. https://doi.org/10.1371/journal.ppat.1002251.
21. Hu Y, Morichaud Z, Perumal AS, Roquet-Baneres F, Brodolin K. 2014. Mycobacterium RbpA cooperates with the stress-response σB subunit of RNA polymerase in promoter DNA unwinding. Nucleic Acids Res 42:10399–10408. https://doi.org/10.1093/nar/gku742.
22. Igarashi K, Fujita N, Ishihama A. 1989. Promoter selectivity of *Escherichia coli* RNA polymerase: omega factor is responsible for the ppGpp sensitivity. Nucleic Acids Res 17:8755–8765. https://doi.org/10.1093/nar/17.21.8755.
23. Lin W, Mandal S, Degen D, Liu Y, Ebright YW, Li S, Feng Y, Zhang Y, Mandal S, Jiang Y, Liu S, Gigliotti M, Talaue M, Connell N, Das K, Arnold E, Ebright RH. 2017. Structural basis of *Mycobacterium tuberculosis* transcription and transcription inhibition. Mol Cell 66:169–179. https://doi.org/10.1016/j.molcel.2017.03.001.
24. Vassylyev DG, Vassylyeva MN, Perederina A, Tahirov TH, Artsimovitch I. 2007. Structural basis for transcription elongation by bacterial RNA polymerase. Nature 448:157–162. https://doi.org/10.1038/nature05932.
25. Murakami KS. 2013. X-ray crystal structure of *Escherichia coli* RNA polymerase sigma70 holoenzyme. J Biol Chem 288:9126–9134. https://doi.org/10.1074/jbc.M112.430900.
26. Boyaci H, Chen J, Lilic M, Palka M, Mooney RA, Landick R, Darst SA, Campbell EA. 2018. Fidaxomicin jams *Mycobacterium tuberculosis* RNA polymerase motions needed for initiation via RbpA contacts. Elife 7:e34823. https://doi.org/10.7554/eLife.34823.
27. Hubin EA, Fay A, Xu C, Bean JM, Saecker RM, Glickman MS, Darst SA, Campbell EA. 2017. Structure and function of the mycobacterial transcription initiation complex with the essential regulator RbpA. Elife 6:e22520. https://doi.org/10.7554/eLife.22520.
28. Minakhin L, Bhagat S, Brunning A, Campbell EA, Darst SA, Ebright RH, Severinov K. 2001. Bacterial RNA polymerase subunit omega and eukaryotic RNA polymerase subunit RPB6 are sequence, structural, and functional homologs and promote RNA polymerase assembly. Proc Natl Acad Sci U S A 98:892–897. https://doi.org/10.1073/pnas.98.3.892.
29. Gentry DR, Burgess RR. 1989. *rpoZ*, encoding the omega subunit of *Escherichia coli* RNA polymerase, is in the same operon as *spoT*. J Bacteriol 171:1271–1277. https://doi.org/10.1128/jb.171.3.1271-1277.1989.
30. Santos-Beneit F, Barriuso-Iglesias M, Fernandez-Martinez LT, Martinez-Castro M, Sola-Landa A, Rodriguez-Garcia A, Martin JF. 2011. The RNA polymerase omega factor RpoZ is regulated by PhoP and has an important role in antibiotic biosynthesis and morphological differentiation in *Streptomyces coelicolor*. Appl Environ Microbiol 77:7586–7594. https://doi.org/10.1128/AEM.00465-11.
31. Richard CL, Tandon A, Sloan NR, Kranz RG. 2003. RNA polymerase subunit requirements for activation by the enhancer-binding protein *Rhodobacter capsulatus* NtrC. J Biol Chem 278:31701–31708. https://doi.org/10.1074/jbc.M304430200.
32. Mukherjee K, Nagai H, Shimamoto N, Chatterji D. 1999. GroEL is involved in activation of *Escherichia coli* RNA polymerase devoid of the omega subunit *in vivo*. Eur J Biochem 266:228–235. https://doi.org/10.1046/j.1432-1327.1999.00848.x.
33. Jacques JF, Rodrigue S, Brzezinski R, Gaudreau L. 2006. A recombinant *Mycobacterium tuberculosis in vitro* transcription system. FEMS Microbiol Lett 255:140–147. https://doi.org/10.1111/j.1574-6968.2005.00071.x.
34. Herrera-Asmat O, Lubkowska L, Kashlev M, Bustamante CJ, Guerra DG, Kireeva ML. 2017. Production and characterization of a highly pure RNA polymerase holoenzyme from *Mycobacterium tuberculosis*. Protein Expr Purif 134:1–10. https://doi.org/10.1016/j.pep.2017.03.013.
35. Banerjee R, Rudra P, Prajapati RK, Sengupta S, Mukhopadhyay J. 2014. Optimization of recombinant *Mycobacterium tuberculosis* RNA polymerase expression and purification. Tuberculosis 94:397–404. https://doi.org/10.1016/j.tube.2014.03.008.
36. Tang H, Severinov K, Goldfarb A, Ebright RH. 1995. Rapid RNA polymerase genetics: one-day, no-column preparation of reconstituted recombinant *Escherichia coli* RNA polymerase. Proc Natl Acad Sci U S A 92:4902–4906. https://doi.org/10.1073/pnas.92.11.4902.
37. Hubin EA, Lilic M, Darst SA, Campbell EA. 2017. Structural insights into the mycobacteria transcription initiation complex from analysis of X-ray crystal structures. Nat Commun 8:16072. https://doi.org/10.1038/ncomms16072.
38. Zuo Y, Steitz TA. 2015. Crystal structures of the *E. coli* transcription initiation complexes with a complete bubble. Mol Cell 58:534–540. https://doi.org/10.1016/j.molcel.2015.03.010.
39. Borukhov S, Goldfarb A. 1993. Recombinant *Escherichia coli* RNA polymerase: purification of individually overexpressed subunits and *in vitro* assembly. Protein Expr Purif 4:503–511. https://doi.org/10.1006/prep.1993.1066.
40. Zhu Y, Mao C, Ge X, Wang Z, Lu P, Zhang Y, Chen S, Hu Y. 2017. Characterization of a minimal type of promoter containing the −10 element and a guanine at the −14 or −13 position in mycobacteria. J Bacteriol 199:e00385-17. https://doi.org/10.1128/JB.00385-17.