Supplementary Information

The structure of bacterial RNA polymerase in complex with the essential transcription elongation factor NusA.

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Far Western Blotting

*B. subtilis* σA was overproduced and purified as described previously (Yang and Lewis, 2008) and employed as the positive control for mapping RNAP interaction by far Western Blotting. All cloning experiments were carried out using *E. coli* DH5α (Table S1).

In order to make radio-labelled probes for far Western blotting, a Kemptide Protein Kinase A recognition sequence (PKA-RS) was inserted between *Not*I and *Acc65*I sites of pNG209 (Yang and Lewis, 2008) using the linker:

5’-GGCCGCAGAATTCCCGGGCTGCGTCGTGCGTCCCTGG-3’ and

5’-GTACCCAGGGACCGACGACCAGCCGCCGGGAATTCTGC-3’ and the resulting vector was termed pNG586 (Table S1). Full length NusA was amplified using the primers:

5’-GTAAATCATATGAGCAGTGAA-3’ (NusA-Forward) and

5’-ACGCAGCCGGGTTCATCCGATTC-3’ (NusA-Reverse) and cloned into pNG586 using the restriction sites *Nde*I and *Xma*I to create pNG592 (Table S1). The N-terminal domain of NusA was amplified using the NusA-Forward and

5’-GCAGCCGAATTCATAAATCACACC-3’ (Reverse) primers and digested with *Nde*I and *EcoR*I and ligated with similarly cut pNG586 to create pNG597 (Table S1). The C-terminal domain of NusA was amplified using the 5’-GTTATTCATATGTATTCTGAATTTA-3’ (Forward) and NusA-Reverse primers and cloned into pNG586 making use of the *Nde*I and *Xma*I restriction sites to create pNG609 (Table S1).

To radiolabel PKA-RS-tagged proteins, 100 µl reactions were assembled that contained 20...
mM Tris-HCl pH7.5, 100 mM NaCl, 12 mM MgCl$_2$, 1 mM DTT and 25 % (v/v) glycerol, 5 mg/ml of target protein, 240 U PKA (Fermentas) and 14 µCi of $^{32}$P-ATP (Perkin-Elmer). Samples were incubated at room temperature for 30 minutes, and the reactions stopped by passage through G-50 micro columns (GE Healthcare) following the manufacturer’s instructions.

Using the *B. subtilis* RNAP homology model (MacDougall *et al.*, 2005; E. Johnston, unpublished), protein fragments were chosen based on domain architecture in order to overproduce individual fragments that would be conducive to correct folding when expressed *in vitro*. The domains chosen in the β subunit consist of amino acids 1-608, 400-760, 750-1040 and 950-1193, while those for β’ comprise amino acids 1-433, 253-610, 600-915 and 800-1199. These fragments, along with full length α, ω$_1$ and ω$_2$ subunits, and RpoB Δβ-flap fragment (750-1040, with an in-frame deletion from amino acids 846-875) were cloned into the pET-based vector (pETMCSIII; Table S1) and overproduced individually in *E. coli* BL21 (DE3) pLysS (Table S1). 20 ml cultures were grown in LB medium supplemented with 100 µg/ml ampicillin at 37°C until the A$_{600}$ reached 0.4 - 0.7, before the addition of IPTG to a final concentration of 0.5 mM and growth for a further 3 hours at 37°C. Cells were harvested in 1 ml aliquots, stored at -80°C and lysed by freeze-thawing when ready to use. Prior to far-Western blotting, the cleared lysates were separated by SDS-PAGE and stained with Coomassie blue to check that overproduction of all fragments had been successful, and so that loading volumes could be adjusted in order that approximately equal amounts of RNAP fragment were present in each lane (supplementary Fig. S2A). For far-Western blots, cell lysates were separated by SDS-PAGE, and transferred onto nitrocellulose membranes. The nitrocellulose membranes were washed 3 times in Tris far Western buffer (TFWB: 20 mM Tris-HCl pH 7.6, 100 mM NaCl, 0.5 mM EDTA, 10 % (v/v) glycerol, 0.1 % (v/v) Tween-20)
before blocking with refold buffer (TFWB with 5 % (w/v) skim milk powder) for 1 hour at room temperature with gentle agitation. The membranes were probed with 30 ml of refold buffer containing 40 nM of prey proteins for 2 hours at room temperature whilst being shaken, before washing 3 times with refold buffer. When radio-labelled probes were used, the membrane was dried and imaged with a phosphorimager (BioRad) according to the manufacturer’s instructions. When probing with σ[^A], the membranes were incubated with a 1:10,000 dilution of rabbit polyclonal anti- σ[^A] in refold buffer, and incubated at room temperature for 1 hour. The membrane was washed in refold buffer before a 1:3,000 dilution of the secondary goat-anti-rabbit horseradish peroxidase (BioRad) antibody was added and incubated for a further 1 hour at room temperature. Detection of the antibody was achieved using the Opti-4CN substrate kit (BioRad) according to the manufacturer’s instructions.

**Affinity-gel chromatography**

RNAP Δβ-flap mutagenesis (removing amino acids 846-875) was achieved using PCR splicing. Two separate PCRs were set up to amplify N- and C-terminal halves of the rpoB gene from plasmid pNG545 (Table S1; Yang and Lewis, 2008) using primers 5’-CAGGTCAACTAGTTCACTGATG-3’ (P1) and 5’-GCCATGAGGCACACAGAAGAGTTCTACAACACAAAAAGATCTCC-3’ (P2), 5’-GGAGATCTTCTTTGTTGGTAATTACACACACTCCGTGCGCCTCATG-3’ (P3) and 5’-GCTAGTTATTGCTCAGCCGG-3’ (P4), respectively. 2 µl of the purified PCR product from both reactions were used as template in the second round of PCR using P1 and P4 to amplify RNAP Δβ-flap. The ~3kb PCR product was digested with *Spe*I and *Xho*I and ligated with similarly cut pNG545 (Table S1; Yang and Lewis, 2008). The resulting vector was named pNG691 (Table S1) and the in-frame deletion was confirmed by DNA sequencing. RNAP
Δβ-flap tip was overproduced using pNG691 and pNG567 (Table S1; Yang and Lewis, 2008), and purified as detailed previously (Yang and Lewis, 2008).

500 pmoles of wt and Δβ-flap RNAP were coupled to 50 µl Affi-Gel 15 resin (BioRad) at 4°C with rotation overnight in 500 µl coupling buffer (10 mM MOPS, pH 8.0). A blank control was also prepared with no protein coupled to the resin. 50 µl of 1 M ethanolamine, pH 8.0 was then added to all three columns to block any free active esters in the resin at 4°C with rotation for 4 h. 50 µl columns were prepared as described by Lewis et al., (1996). Following washing with 1 ml binding buffer (10 mM Tris, 150 mM NaCl, pH 7.8), 3000 pmoles of NusA or NusA NTD was equilibrated with the different columns at RT for 4 h. The columns were then washed with 1 ml binding buffer and NusA/NusA NTD was eluted with 400 mM NaCl, 10 mM Tris, pH 7.8. Anti-NusA Western blotting was performed as described by Davies et al (2005).

**In vitro reconstitution of NusA-RNAP complex**

To reconstitute NusA-RNAP complexes for structural study, RNAP isolated to homogeneity from native *B. subtilis* cells (BS200; Yang and Lewis, 2008) was mixed with purified recombinant NusA (Davies et al., 2005) at a molar ratio of 1:3 in dilution buffer (20 mM Tris pH 7.8, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT). RNAP core or the reconstituted NusA-RNAP complex was loaded onto a Sepharose 6 column (GE Healthcare) in dilution buffer at a flow rate of 0.5 ml/min. The absorbance profile of each sample was monitored at 280 nm. When the sample containing only RNAP core was analysed, only one peak centred at an elution volume of 14 ml was observed (Fig S3A). This peak occurred at the same position in the UV profile of the reconstituted complex (Fig S3C). Since NusA only contributes 10 % to
the mass of the reconstituted complex, no shifts in the elution volume would be expected under the conditions of gel filtration. The peak fractions were pooled, separated by SDS-PAGE and assessed by Western blotting using rabbit polyclonal anti-RpoC and anti-NusA antibodies (Davies et al., 2005). An immunoreactive band at the size of NusA (~42 kDa) was only present in the reconstituted complex (compare Fig. S3B and D). A minor peak at 17 ml in the elution profile of the NusA-RNAP complex corresponded to unbound excess NusA, and was confirmed by Western blotting (not shown). Taken together, these data verified that NusA was successfully incorporated into the complex, and that the complex was stable at room temperature on gel filtration, making it suitable for structural study by transmission electron microscopy.

**Electron microscopy and image processing**

The reconstituted NusA-RNAP complex and RNAP core sample were diluted to 0.08 µM and 4 µl of sample was applied to home-made continuous carbon grids for 30 s before washing three times with dilution buffer. The specimens were then stained with a 1 % (w/v) uranyl acetate aqueous solution. Negative stain samples were imaged on a Tecnai F30 FEG TEM (FEI Company) operated at 300 keV at a magnification of 59,000 x at room temperature. Images were taken using low dose settings (~60 e/ Å²) at a defocus of 600-900 nm and recorded on a GATAN 4k x 4k CCD camera (Gatan Inc.) with 2x binning resulting in a pixel size corresponding to 3.9 Å at the specimen level. 240 and 524 micrographs were taken for the RNAP core and the NusA-RNAP complex, respectively. Particle picking was performed manually using SwarmPS software (Woolford et al., 2007) and both of the data sets were manually checked and edited three times. 12,622 (RNAP core) and 15,027 (NusA-RNAP complex) particles were used for image processing.
The EMAN software package v1.8 (Ludkte et al., 1999) was used for 3D image processing and reconstructions. Individual particles were appended, centre aligned, and boxed into 72 x 72-pixel images. A Gaussian sphere was employed as an initial model. The RNAP core dataset was band-pass filtered to 52-210 Å and after 26 iterations the model converged to about 40 Å as judged by Fourier-shell correlation. The 26th and 27th models were filtered to 50 Å resolution and used as initial models for the multirefine routine in EMAN, with the RNAP core dataset filtered to 13 Å. After another 39 iterations one of the models converged stably to about 30 Å, which was employed as the starting model for both RNAP core and NusA-RNAP complex reconstruction. The final reconstructions, after 28 iterations, are estimated to have resolutions of 25 and 22 Å, respectively (Fig S4A). A separate lower resolution reconstruction of RNAP core performed using Imagic (van Heel et al., 1996) comprising ~9000 particles (not shown) resulted in a similar structure with identical dimensions to the core reconstruction using EMAN showing that two independent single particle analysis approaches produced the same result.

**Nanogold labelling**

20 pmoles of RNAP isolated from BS200 (Table S1) were mixed with 200 pmoles of Ni-NTA nanogold probe (Nanoprobes) in 200 µl buffer (10 mM Tris, 150mM NaCl, pH 7.8) at 4°C overnight to label the 6xHis-tagged C-terminal domain of the β’ subunit. The sample was then applied to home made carbon grids, washed 4 times with water and stained with 1% (w/v) uranyl formate. Images were acquired as detailed above and 964 nanogold-labelled particles picked. These particles were then appended with the unlabeled RNAP particle set (12,622 particles) and a reconstruction was performed using the RNAP core electron density map as an initial model. After 8 rounds of iteration, 440 class averages were calculated of
which 17 had visible nanogold particles. Due to the location of the C-terminus of the $\beta'$ subunit, many labelled particles lay with an orientation that precluded unambiguous identification due to the negative stain ring. Therefore, only particles in which the gold could be positively identified as a regular dark spot within the stain ring were selected, resulting in the number of class sums (17/440) containing gold-labelled particles. These class averages were then compared with corresponding class averages from the unlabeled dataset and the position of the nanogold mapped onto the projections/3D views.

**Docking**

A homology model of *B. subtilis* RNAP EC (RNAP EC) was made by submitting multiple sequence alignments of each subunit to the SWISS-MODEL server (Guex and Peitsch, 1997; http://swissmodel.expasy.org) using the ‘alignment interface’ and the crystal structure of the *Thermus thermophilus* RNAP EC (Vassylyev et al., 2007) as a template. The full enzyme was assembled using the individually modelled subunits and DNA and RNA chains from the template were subsequently added to this model.

NusA was similarly modelled using the crystal structures of *Thermotoga maritima* NusA (Worbs et al., 2001) and the C-terminal domain from *M. tuberculosis* in complex with RNA (Beuth et al., 2005) as the templates.

Normal mode analysis (NMA) was performed on the RNAP EC homology model in the absence of DNA/RNA using the web-based program eLNemo (Suhre and Sanejouand, 2004; http://www.igs.cnrs-mrs.fr/elnemo/).
Mapping of the nanogold-labelled C-terminal domain of the β’-subunit confirmed the orientation of RNAP molecule relative to the electron density map. The RNAP EC model was docked accordingly into the negative stain electron density map using UCSF Chimera (Pettersen et al., 2004) and manually refined to achieve the best fit. The goodness-of-fit was estimated using SITUS (Chacón and Wriggers, 2002) with electron density envelopes set at 25 Å.

NusA was docked as two separate rigid domains into the extra mass evident in the NusA-RNAP complex. The 25 amino acid linker region between the two domains of NusA (residues 124 and 148) was not modelled. The docking of the individual domains of NusA into the reconstructed electron density map resulted in a greater separation between the N- and C-terminal domains (38 Å) in the NusA-RNAP complex than has been observed in crystal structures of isolated NusA (23 Å). Given the linker region between domains is 25 amino acids, such an increase in domain separation is not unreasonable. The N-terminal domain of NusA (residues 1-123) was docked into the mass around the β flap tip region and manually refined. The crystal structure of the C-terminal domain of M. tuberculosis NusA in complex with RNA was used to obtain an homology model of the C-terminal domain of B. subtilis NusA (Beuth et al., 2005) and was employed as a reference to determine the RNAP interacting face and the rotational orientation with respect to the RNA exit channel to dock the C-terminal domain of NusA (residues 149-343) into the remaining mass.

**Model quality**

To determine the correct handedness, maps of both enantiomers of each structure were produced and the ones enabling the RNAP homology model to be docked were chosen. The resolution of the 3-D reconstructions was estimated by the even-odd (EO) test of EMAN, in
which two independent reconstructions were performed from the even and odd halves of the dataset and compared using a Fourier shell correlation (FSC) curve (Ludkte et al., 1999; Fig S4A). The conservative 0.5 FSC criterion was applied and the resolution was determined to be just below 25 Å for the RNAP core and 22 Å for the NusA-RNAP complex (Fig. S4A). The number of particles within each class for the final reconstructions followed a typical Gaussian distribution with the peaks centred at around 8-16 particles per class (Fig. S4B). Since a uniform angular sampling algorithm is employed in EMAN, the particle distribution reflected the random orientation of the molecules on the grids. For each structure the entire datasets were classified into 1,174 class averages and a total of 12,586 particles for the RNAP core and 14,725 for the NusA-RNAP complex were incorporated into the final round of reconstruction. The remaining particles were discarded based on their poor matching with the projections, which served as the references for particle classification. Also, there was excellent agreement between 3D reconstructions, reprojections and their corresponding class averages indicating that Euler angle assignment was correct (see Fig. 2A). A comparison of the RNAP core model projections with their counterparts in the NusA-RNAP complex, reveals that the majority of them are indistinguishable (Fig 2A), whilst at other angles the additional mass corresponding to NusA could only be observed in the projections from the NusA-RNAP complex (Fig 2A; arrows). Finally, individual particles within a class were morphologically similar to the class averages and reprojections, which suggested the classification was accurate (Fig. S4C).

The El Nemo Elastic Network Model server was used to carry out Normal mode analysis for the low frequency modes of the RNAP structure to assess protein flexibility in an attempt to optimise the fit of the model to the EM derived molecular envelope. The PDB file for B. subtilis RNAP core homology model was uploaded to the El Nemo server (Suhre and
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Sanejouand, 2004) for calculation of normal modes. Normal modes 7 and 8 showed significant movements in the 'claw' region which is thought to be the most flexible part of the protein (Darst et al., 2002; Van Wynsberghe et al., 2004). Both the original PDB and the 'fully open' form were docked in the EM envelope (Fig. S5; M1) and clearly fit within the electron density, even in the most open form. Correlation coefficients of goodness of fit were calculated with Situs (Chacón and Wriggers, 2002) and gave values of 58.4% for the fully open structure and 58.2% for the closed structure (see Fig. S5).
Suplementary Figure Legends

Figure S1. Sequence alignments of NusA and RNAP β-flap. Sequences were aligned and secondary structures annotated according to the structures present in the B. subtilis NusA and RpoB homology models. Red/orange rods, α-helices; blue arrows, β-sheet. Panel A: complete NusA sequences were aligned and the black dots demark domain boundaries. Panel B: β-flap sequences were aligned to amino acids 761-949 of the B. subtilis β subunit and the region corresponding to the deletion (Del.) made in β-flap tip mutant fragments and RNAP denoted by the black arrows. A ribbon figure of the structure of the aligned β-flap sequence is shown below the alignment with the region corresponding to the deletion coloured green.

Efaec, Enterococcus faecalis (aa 761-949); Spneum, Streptococcus pneumoniae (aa 757-945); Saure, Staphylococcus aureus (aa 760-948); Cdiff, Clostridium difficile (aa 773-961); Ecoli, Escherichia coli (aa 802-990); Paeru, Pseudomonas aeruginosa (aa 807-995); Bsubt, Bacillus subtilis (aa 761-949).

Figure S2. Overproduction of RNAP subunits and fragments and far-Western blotting with the NusA C-terminal domain. Panel A, strains containing plasmids for the overproduction of RNAP subunits and fragments were grown and overproduction induced as detailed in supplementary methods. Following lysis and clarification, cleared lysates were loaded onto polyacrylamide gels. The fragments and subunits loaded are indicated above the lanes. \(\omega_1\) (YkzG) and \(\omega_2\) (YloH) are small and run close to the dye front at approximately 10 kDa. The \(\Delta\beta\)-flap fragment corresponds to β fragment 750-1040 in which amino acids 846-875 have been deleted (see text for details). Panel B, far-Western blotting of RNAP subunits and fragments using \(^{32}\)P-labelled NusA C-terminal domain. Due to the very low level of signal obtained, image contrast has been enhanced in order that the weak binding of NusA C-
terminal domain to RNAP β and β’ subunit fragments can be observed. The fragments and subunits loaded are indicated above the lanes.

**Figure S3. Reconstitution of the NusA-RNAP complex.** (A) UV absorbance chromatogram of the RNAP core and (C) the reconstituted NusA-RNAP complex. The peaks centered at around 14 ml were pooled and analyzed by Western blotting with anti-RpoC and anti-NusA antibodies. The ~40 kDa immunoreactive band of NusA was only present in the reconstituted complex (D), and not observed in the RNAP core sample (B). The membrane was cut around the 70 kDa level in order to perform RpoC and NusA blots, resulting in the line at this point on reassembly of the sample lanes (B and D).

**Figure S4. Assessment of model quality.** (A) Estimation of the resolutions of the RNAP core and the NusA-RNAP complex by the EMAN E/O test and Fourier shell correlation. (B) Particle distribution of the RNAP core and the NusA-RNAP complex. (C) Reprojections (I), class averages (II), and individual picked particles from the class averages (III) of RNAP core and NusA complex classes showing accurate assignment of particles to classes. The orientations for core and complex are the same for each set of reprojections, and in the bottom panel the additional mass due to NusA in the complex is indicated by a red arrow. Particles (III) are have been extracted in the same orientation as they were used to create the class sums.

**Figure S5. Docking of the RNAP core model into the experimental EM map.** Normal mode analysis indicated that the claw formed by β and β’ subunits is highly flexible without DNA. When docked in the two extreme conformations (closed and open claw), the RNAP homology models fit equally well into the reconstructed map.
M1. Normal mode analysis of RNAP core homology model with the electron density map of the RNAP core negative stain reconstruction. The electron density map is shown as a brown mesh and RNAP core homology model as a green ribbon with structures in the same orientation as those labelled in supplementary Fig S5.

M2. Comparison of RNAP core and NusA-RNAP complex reconstructions. RNAP core is shown in light grey as a solid surface in which the β flap has been coloured green. The NusA-RNAP complex is shown as a white mesh with the β flap coloured green, NusA N-terminal domain in yellow and C-terminal domain in red.
Table S1. Strains and plasmids used in this study

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Yang et al. Structure of NusA-RNA polymerase complex.

9xHis-T$_\psi$

bla $P_{\psi10}$PKARS-

This work. PKA recognition site cloned in

'6xHis-T$_\psi$

Ndel-Acc65I sites of pNG209

bla $P_{\psi10}$ -NusA-

This work. Full length NusA gene cloned in

PKARS-6xHis-T$_\psi$

Ndel-XmaI sites of pNG586

bla $P_{\psi10}$ -NusANTD-

This work. NusANTD gene (aa 1-127) cloned in

PKARS-6xHis-T$_\psi$

Ndel and EcoRI sites in pNG586

bla $P_{\psi10}$ -NusACTD-

This work. NusACTD gene (aa 127-371) cloned

PKARS-6xHis-T$_\psi$

in Ndel and XmaI sites in pNG586

bla $P_{\psi10}$ - 6xHis-

This work. rpoB gene fragment (aa 1-608)

rpoBaa1-608-T$_\psi$

cloned in Ndel-EcoRI sites of pETMCSIII

bla $P_{\psi10}$ - 6xHis-

This work. rpoB gene fragment (aa 400-760)

rpoBaa400-760-T$_\psi$

cloned in Ndel-EcoRI sites of pETMCSIII

bla $P_{\psi10}$ - 6xHis-

This work. rpoB gene fragment (aa 750-1040)

rpoBaa750-1040-T$_\psi$

cloned in Ndel-EcoRI sites of pETMCSIII

bla $P_{\psi10}$ - 6xHis-

This work. rpoB gene fragment (aa 950-1193)

rpoBaa950-1193-T$_\psi$

cloned in Ndel-EcoRI sites of pETMCSIII

bla $P_{\psi10}$ - 6xHis-

This work. rpoC gene fragment (aa 1-433)

rpoCaa1-433-T$_\psi$

cloned in Ndel-EcoRI sites of pETMCSIII

bla $P_{\psi10}$ - 6xHis-

This work. rpoC gene fragment (aa 253-610)

rpoCaa253-610-T$_\psi$

cloned in Ndel-EcoRI sites of pETMCSIII
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This work. \( rpoC \) gene fragment (aa 600-915) cloned in \( Nde\text{-}EcoRI \) sites of pETMCSIII

This work. \( rpoC \) gene fragment (aa 800-1199) cloned in \( Nde\text{-}EcoRI \) sites of pETMCSIII

This work. \( rpoB \) gene fragment (aa 750-

\( rpoB\) aa750-1040\(\Delta\)846-875) cloned in \( Nde\text{-}EcoRI \) sites of pETMCSIII

This work. pNG545, in which amino acids 846-875 of the \( rpoB \) gene have been deleted.

\( P_{\text{15A}} \)

\( bla, \) cat, ampicillin and chloramphenicol resistance genes respectively; \( P_{\psi10} \) phage T7 promoter; \( P_{\psi6\text{His}} \) phage T7 promoter with lacO site; \( T_\psi \), T7 transcription terminator.

**Supplementary References.**


GraphModel. 23:297-303


Supplementary Figures

FigS1
Fig S2

A

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MW (kDa)

10 25 35 40

B

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Image showing protein bands with molecular weight markers.
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FigS3
Yang et al. Structure of NusA-RNA polymerase complex.

FigS4

A

B

C

RNAP

I

II

complex

I

II

FigS5

Closed

Open

α

β

β'

α