Mycobacterium RbpA cooperates with the stress-response σ^B subunit of RNA polymerase in promoter DNA unwinding

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Supplemental Experimental Procedures

M. tuberculosis core RNAP purification.

Co-overexpression of the recombinant *M. tuberculosis* RNAP core enzyme was performed in *E. coli* BL21(ED3) cells. The 1l cell culture transformed with the pMR4 plasmid was induced by 1mM of Isopropyl β-D-thiogalactopyranoside (IPTG) at OD_{600} ~ 0.6. and grown at 24 °C for 20 h. Bacteria were collected by centrifugation at 4000 g for 30 min (4 °C) and resuspended in 100 ml of lysis buffer (40 mM Tris-HCl pH 7.9, 500 mM NaCl, 5% Glycerol, 2 tablets of protease inhibitors (Roche), 0.2 mg/ml lysozyme, and 0.2 mM β-mercaptoethanol). Cells were disrupted by sonication, and the lysate was cleared by centrifugation (10,000 g, 30 min, 4 °C) and filtration through 0.45 µm filter before loading to 5 ml HisTrap FF column (GE Heathcare) equilibrated with 40 mM Tris-HCl pH 7.9, 500 mM NaCl, 5% Glycerol, 0.2 mM β-mercaptoethanol, 20 mM Imidazole buffer. RNAP core was eluted in the buffer: 40 mM Tris-HCl pH 7.9, 500 mM NaCl, 5% Glycerol, 0.2 mM β-mercaptoethanol, 300 mM Imidazole. The RNAP core samples were dialyzed overnight against Buffer A (20 mM Tris-HCl pH 7.9, 5% Glycerol, 0.2mM β-mercaptoethanol, 0.2 mM EDTA). The samples were then loaded on 20 ml HiPrep Heparin FF column (GE Heathcare). After being washed with 30% of Buffer B (20 mM Tris-HCl pH 7.9, 1 M NaCl, 5% Glycerol, 0.2 mM β-mercaptoethanol, 0.2 mM EDTA), RNAP core was eluted in 60% of Buffer B. The samples were diluted with 1 volume of Buffer A, concentrated by Ultracel-100 membrane filter unit (Millipore)

and loaded to Superose 6 column (GE Healthcare) equilibrated in Buffer S (20 mM Tris-HCl pH7.9, 300 mM NaCl, 5% Glycerol, 0.2 mM β-mercaptoethanol, 0.2 mM EDTA). The purified RNAP core was supplemented with 2 mM $MgCl_2$ and 1 μM $ZnCl_2$, concentrated to 8 mg/ml and stored at -80 °C in 50% glycerol.

Purification of the σ subunits and RbpA

Genes encoding *M.tuberculosis* σ subunits were amplified from the H37Rv (σ^B to σ^E and σ^G to σ^L) or H37Ra (σ^M) genomic DNA and cloned into pET21a or pET28a vectors. The constructed plasmids are listed in Table S1. The pSR01 plasmid coding for σ^A and pSR5 plasmid coding for σ^F were a generous gift from Dr Sébastien Rodrique (1). The σ subunits were expressed in *E. coli* BL21(ED3) and purified from soluble fraction (σ^A, σ^C and σ^H) or from insoluble fraction by Ni^{2+}-agarose affinity chromatography. *M. tuberculosis* RbpA protein was purified as described before (2).

Table S1. Plasmids used in the current study.

Name	Description	Source
pSR52	pET16b derivative containing the *rpoA* gene encoding RpoA	Dr Rodrique
pSR01	pET30a derivative containing the Rv2703 gene encoding SigA	Dr Rodrique
pSR05	pET30a derivative containing the Rv3286c gene encoding SigF	Dr Rodrique
pET-SigB	pET28a derivative containing the Rv2710 gene encoding SigB	This study
pET-SigC	pET21a derivative containing the Rv2069 gene encoding SigC	This study
pET-SigD	pET21a derivative containing the Rv3414c gene encoding SigD	This study
pET-SigE	pET21a derivative containing the Rv1221 gene encoding SigE	This study
pET-SigG	pET21a derivative containing the Rv0182c gene encoding SigG	This study
pET-SigH	pET21a derivative containing the RV3223c gene encoding SigH	This study
pET-SigI	pET21a derivative containing the Rv1189 gene encoding SigI	This study
pET-SigJ	pET21a derivative containing the Rv3328c gene encoding SigJ	This study
pET-SigK	pET28a derivative containing the Rv0445c gene encoding SigK	This study
pET-SigL	pET21a derivative containing the Rv0735 gene encoding SigL	This study

pET-SigM	pET21a derivative containing the MRA_3950 gene encoding SigM	This study

Table S2. Sequences of the oligonuclotides used in the current study

Name	Sequence 5'-3'	Description
PrpoZ-F	AGTGGATCCAAGAAGGAGATATAGATATGAGTATCTCGCAGTCCG	pMR4 construction
PrpoZ-R	TATGTCGACTCAGCTACTCGCCCTCGGTGTGCTC	
PrpoB-F	TTAAAGCTTAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATCT AGAAGGAGATATAGATATGGCAGAT	
PrpoB-R	CTAGAATTCAGAGAATTACGCAAGATCCTCGACACTTG	
PrpoC-F	ACAGAATTCAGAAGGAGAcagctATGCTCGACGTCAACTTCTTC	
PrpoC-R	ATAGATATCTCAGTGGTGGTGGTGGTGGTGGCGGTAGTCGCTGTAGCCGTAG TC	
PsigB-NF	TTACATTCATATGGCCGATGCACCCAC	pET-SigB construction
PsigB-XR	AAACTCGAGTTAGCTGGCGTACGACCGCA	
PsigC-NF	TTTTTTCATATGACCGCGACGGCAA	pET-SigC construction
PsigC-SR	TTTGTCGACGCCGGTGAGGTCGTC	
PsigD-NF	TTTTTTCATATGGTCGATCCGGGAGT	pET-SigD construction
PsigD-XR	ACACTCGAGCGCATAGTCACCTGC	
PsigE-NF	GGAATTCCATATGGAACTCCTCGGCGGACCC	pET-SigE construction
PsigE-XR	CCGGCTCGAGTGCGCGAACTGGGTTGAC	
PsigG-NF	TTTTTTCATATGCGCACATCGCCGATG	pET-SigG construction
PsigG-XR	AAACTCGAGCAGCGAATCGGGCAGG	
PsigH-NF	TGTCTTCATATGGCCGACATCGATG	pET-SigH construction
PsigH-XR	AATCTCGAGTGACGACACCCCCTCG	
PsigI-NF	TTGGTTCATATGTCGCAACACGACCC	pET-SigI construction
PsigI-NR	AAAGCGGCCGCACCGCCGCCGAGTTCG	
PsigJ-NF	TGGGGTCATATGGAGGTTTCCGAAT	pET-SigJ construction
PsigJ-XR	AAACTCGAGATTCCGGTGATGCCTG	
PsigK-NF	TTTTTTCATATGACCGGACCGCCAC	pET-SigK construction
PsigK-XR	AATCTCGAGTTATGACACGTCCAGGCAG	
PsigL-NF	TTTTTTCATATGGCTCGTGTGTCGGG	pET-SigL construction
PsigL-XR	AACCTCGAGTCGAGTAACTCCCAGTT	
PsigM-NF	ATTTCTCATATGCCGCCACCGATTG	pET-SigM construction
PsigM-XR	AAACTCGAGGCGCCGGATGTTCACC	
PsigAp-F*	CACTGAAACTTGCCCGCTC	*sigA*P amplification
PsigAp-R	GCGTTGATATCAGCGTGAAT	
PctpBp-F*	CGCCCGTGCATCCAGCGGGCCATAG	*ctpB*P amplification
PctpBp-R	GGCCCTCCTCGGATCGGCGTATTTG	
PsigDp-F*	GCCCGGTGGATGCTGGCGTACC	*sigD*P amplification
PsigDp-R	CGCGCGTTACGAAGCGGACACATC	
PsigBp-F*	TCAGGACTTTCTCAGGTCTTCG	*sigB*P amplification

PsigBp-R	CCCTTGTGGGTGCATCGGCC	
PsigEp-F*	CGGCGGACCCCGGGTTGGG	*sigE*P amplification
PsigEp-R	GCGCGCTGCAATAAGTTGGCAAGTC	
PsigIp-F*	GCGCCGAGCGCTGAAATCGCCGGT	*sigI*P amplification
PsigIp-R	GCCCTCCCGCCGACCCATGCTGG	
PsigLp-F*	GCGCGGCTGCCGGCGCGGGCAGTG	*sigL*P amplification
PsigLp-R	GCGGCGCCCGACACACGAGCCAC	
PrrnAp-F*	ATCGAACGGGTATGCTGTTAG	*rrnA*P amplification
PrrnAp-R	TTGAGTTCTCAAACAACACGCT	
PrpfCp-F*	CCCGATCTCACCACTCTCTCTAATC	*rpfC*P amplification
PrpfCp-R	GCGGCCATGGCGGACTTGATGAG	
PusfXp-F*	AAGTCCGGCGGCAGCGG	*usfX*P amplification
PusfXp-R	CCTGCTGGTAGTGGGGATCT	
PlexAp-F*	CCGGCGTCATGTTCGCTCCTCCAAC	*lexA*P amplification
PlexAp-R	GCGCGTGCGACACGCTTCGATC	
PesxUp-F*	GCGGTTGAACCGGGAAGGATCGGAG	*esxU*P amplification
PesxUp-R	GGGCGAAGTCGGCGTTCAGCGTGTT	
galPAAtop-Cy3	GTTTATTCCATGTCACACTTTTCGCATCTTTTCGTTGCTATAATTATTTCATACCA AAAGCCTAATGGAGCG	*gal*P1$_{AA}$ assembly
galPAAbottom	CGCTCCATTAGGCTTTTGGTATGAAATAATTATAGCAACGAAAAGATGCGAAAA GTGTGACATGGAATAAAC	
sigAP-13Cforw	CACTGAAACTTGCCCGCTCGGGCTGTACTCGTGCGCAGTAAAGCTACAATGGT CAGCGGCGGC	*sigA*P^{mut} T$_{-13}$ → C
sigAP-13Crev	GCGTTGATATCAGCGTGAATACTCGCGCGCTATCGGTCGGGCGGCCGCCGCT GACCATTGTAGCTTTACTGCG	
sinP3-13Tforw	GGTAAACAAAAAGTCTGCTCATCCTGGTCCGGCAGCCAGAAGTCATACCGTAA ATCCTTTCTGAATGTGTTATAATATCAC	*sin*P3^{mut} C$_{-13}$ → T
sinP3-13Trew	ACGGTATTGTTTAATACGCTGGCCAATCAATGTCATCACCTTCCTTGTGATATTA TAACACATTCAGAAAGGATTTACGG	

* oligonuclotides labelled at the 5'-end by fluorescein.

Table S3. *Mycobacterium* promoters used in the current study

Promoter	Recognizing σ subunit	Reference
*rrnA*P3	σ^A, σ^B	(3)
*sigA*P	σ^A, σ^B	This study
*ctpB*P	σ^C	(4)
*rpfC*P	σ^D	(5)
*sigB*P	σ^E	(6)
*usfX*P1	σ^F	(7)
*lex*AP	σ^G	(8)

*sigE*P2	σ^H	(9)
*sigI*P	σ^I, σ^J	(10)
*mptC*P	σ^K	(11)
*sigL*P	σ^L	(12)
*PesxU*P	σ^M	(13)

Supplemental Figure Legends

Figure S1. (**A)** Flow chart of the pMR4 plasmid construction. Primers used in each PCR step are listed in the Table S2 and the primer names are marked on the figure under "PCR". Restriction enzymes used for the digestion reactions are marked under "Digestion". (**B**) Map of the pMR4 plasmid. (**C**) Expression of the *M. tuberculosis* RNAP core enzyme in *E. coli* BL21(ED3) at 24 °C analyzed by using 13% SDS-PAGE. (**D**) Purified *M. tuberculosis* RNAP core enzyme and σ factors resolved using 13% SDS-PAGE.

Figure S2. (**A**) The [^{32}P]-RNA run-off products synthesized in the multiple-round transcription assay from the *sigA*P and *rrnA*P3 promoters. Transcription was carried by the RNAP holoenzymes containing σ^A and σ^B subunits either in the presence or absence of RbpA. Quantification of the bands presented as bar graph is shown below each panel. (**B-M**) Effect of RbpA addition on the RNAP-promoter complex formation. EMSA of the promoter complexes formed by the RNAP holoenzymes containing the indicated σ subunits either in the presence or absence of RbpA. The names of promoter DNA fragments used in each experiment are indicated above the panels. **(N)** The C-terminus of RbpA is required for promoter complex formation. EMSA of the σ^A-RNAP with the *B.sabtilus sin*P3 promoter performed either in the absence or presence of the full length RpbA (FL) or the mutant RbpA (ΔC) with the deletion of the C-terminal residues 80-111 (14). **(O)** EMSA of the σ^B-RNAP complexes formed with the *lac*UV5 and *gal*P_{AA} promoters in the presence and absence of RbpA. **(P)** $KMnO_4$ probing of the complexes of σ^B-RNAP with the *gal*P_{AA} promoter. The sequence

of the non-template strand of the *gal*P_{AA} promoter -10 element is shown above the panel. Black triangles indicate thymines of the transcription bubble reactive to $KMnO_4$.

Figure S3. (A) EMSA of the *lac*UV5 promoter complexes formed by hybrid RNAP holoenzymes assembled with the indicated σ subunits. Mtb – *M.tuberculosis*, Eco – *E.coli*. Core RNAP was at 200 nM, σ subunits at 500 nM, *lac*UV5 promoter at 50 nM. **(B)** Alignment of the σ subunit regions 2 and 4. Mtb – *M.tuberculosis*; Eco – *E.coli*; Taq – *Thermus aquaticus*. Boundaries of the subregions are indicated by arrows. Most conserved residues known to interact with the extended -10 and -35 elements according to genetic and structural studies are indicated (15-18).

Figure S4. (A) Nucleotide sequences of the *sin*P3 and *sigA*P promoter regions comprising the -10 element shown in bold. Substitutions introduced at the promoter position -13 are indicated by vertical arrows. Transcription start site (+1) is in bold italic. **(B)** EMSA of the σ^B-RNAP complexes formed with the wild type and the mutant promoter DNA fragments. **(C)** The [^{32}P]-RNA run-off products synthesized in the multiple-round transcription assay from the wild type and mutant *sin*P3 and *sigA*P promoter templates. **(D)** Quantification of the RNA products shown on panel C presented as bar graphs. Values were normalized to the signal obtained for the wild type template in the presence of RbpA.

Supplemental References

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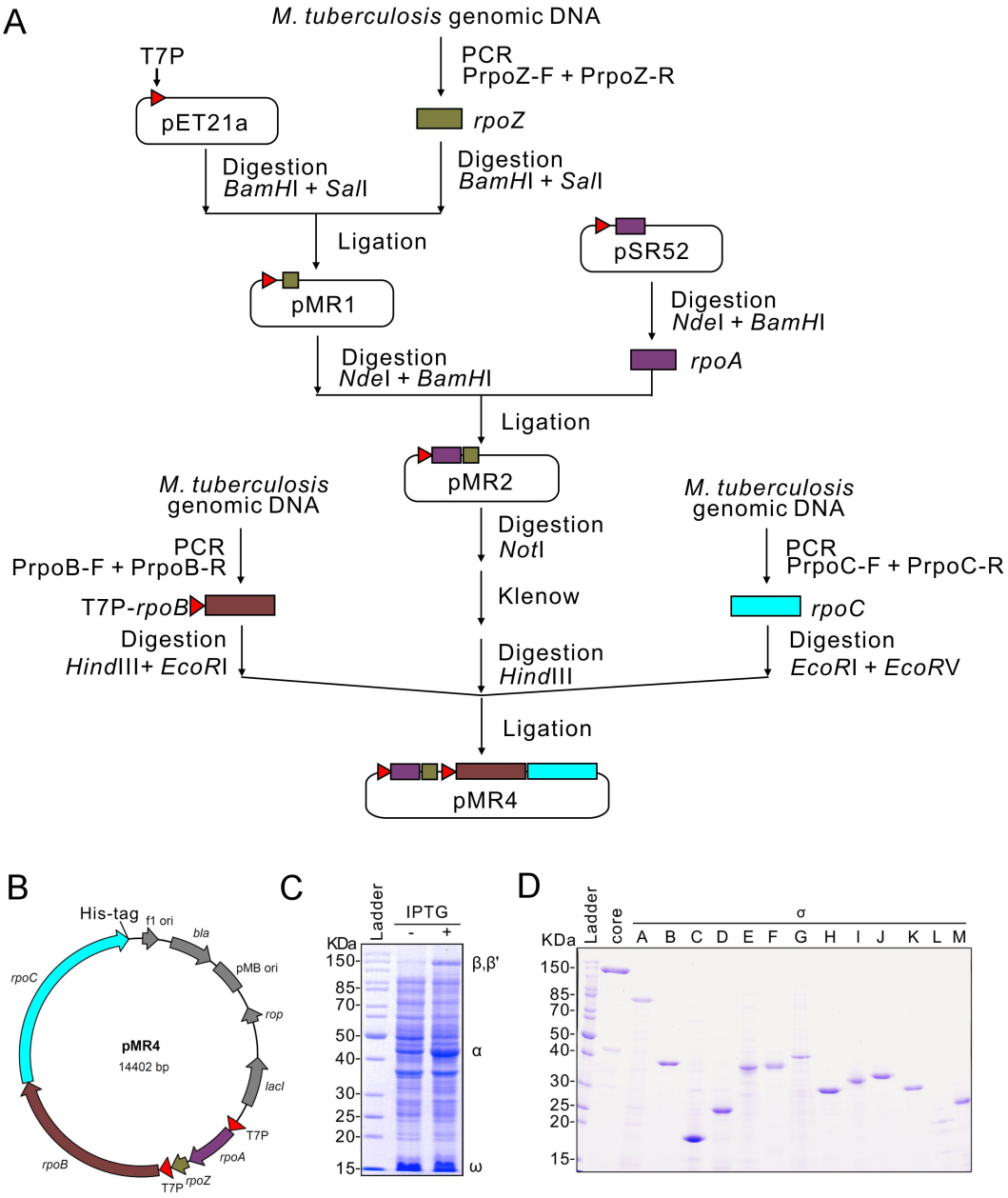
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rpoZ
Digestion
BamHI + SalI
Digestion
BamHI + SalI
Ligation
pSR52
pMR1
Digestion
NdeI + BamHI
Digestion
NdeI + BamHI
rpoA
Ligation
pMR2
M. tuberculosis genomic DNA
M. tuberculosis genomic DNA
PCR
PrpoB-F + PrpoB-R
Digestion
NotI
PCR
PrpoC-F + PrpoC-R
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HindIII
Digestion
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Figure S1

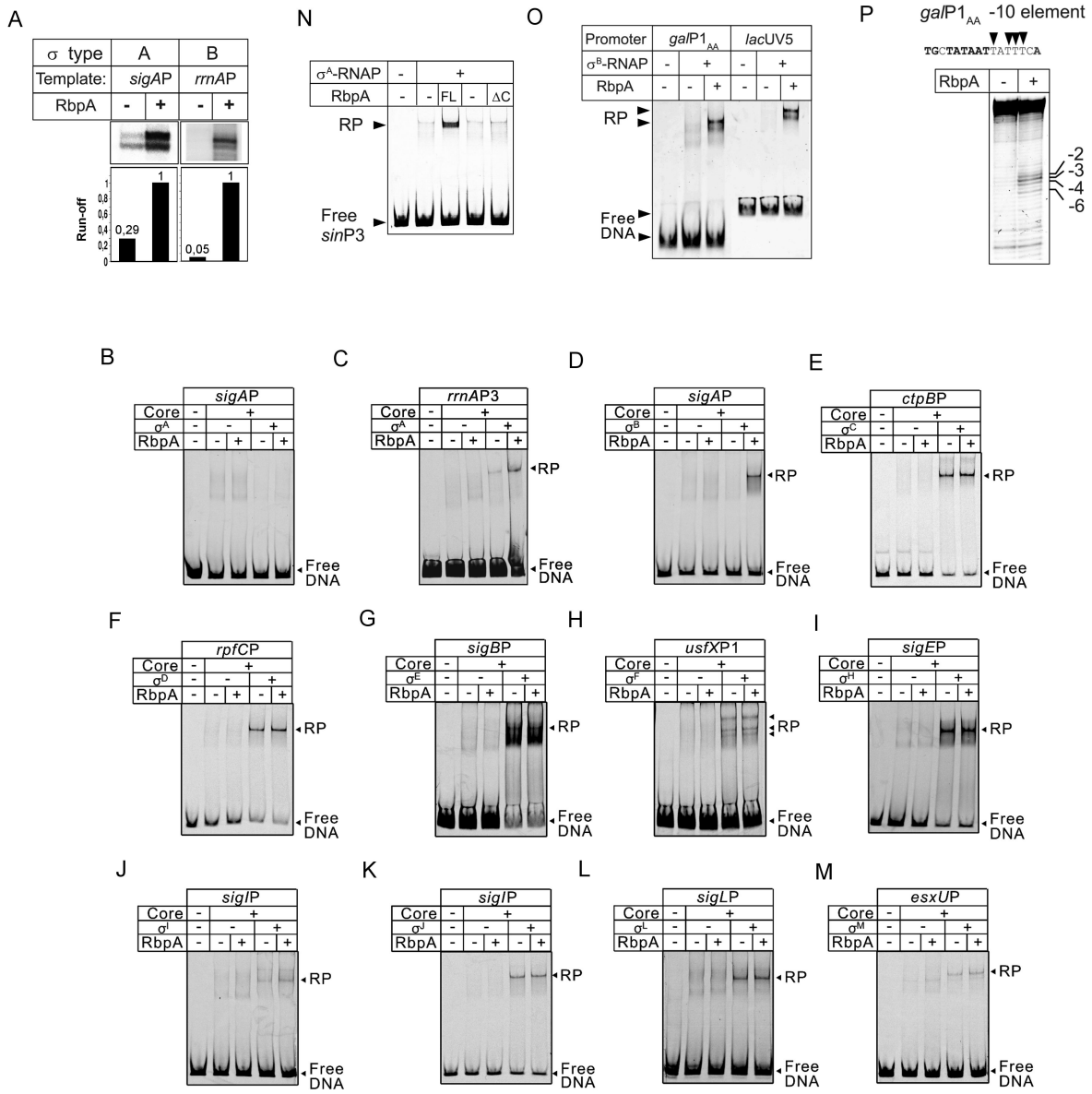
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Figure S2

A

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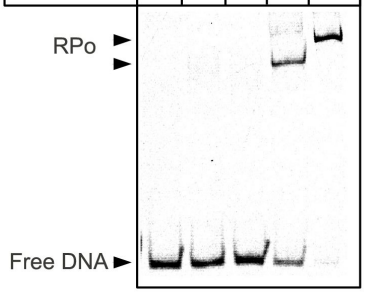
σ^{B}	-	+	-	+	-



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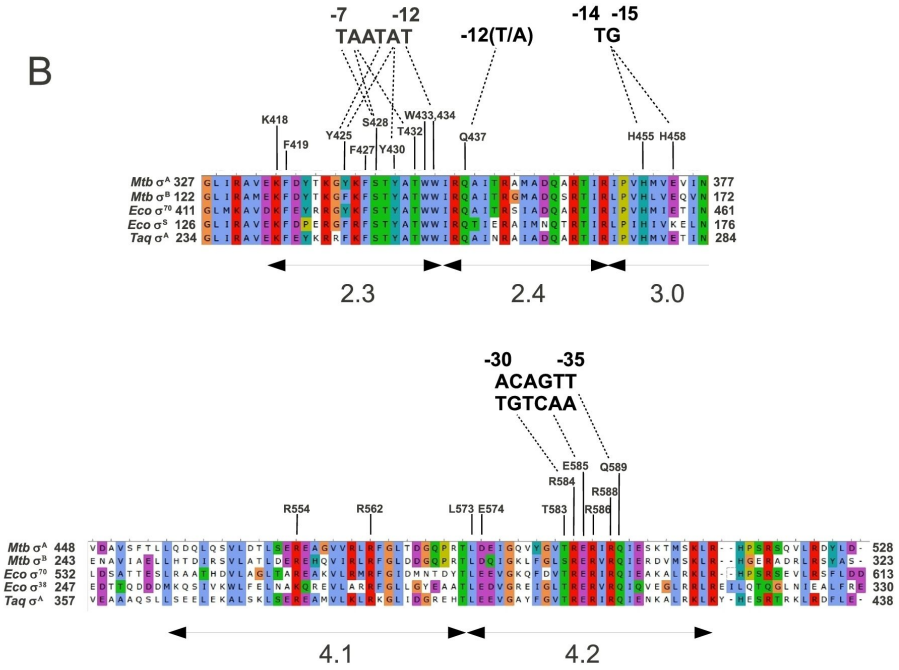

Figure S3

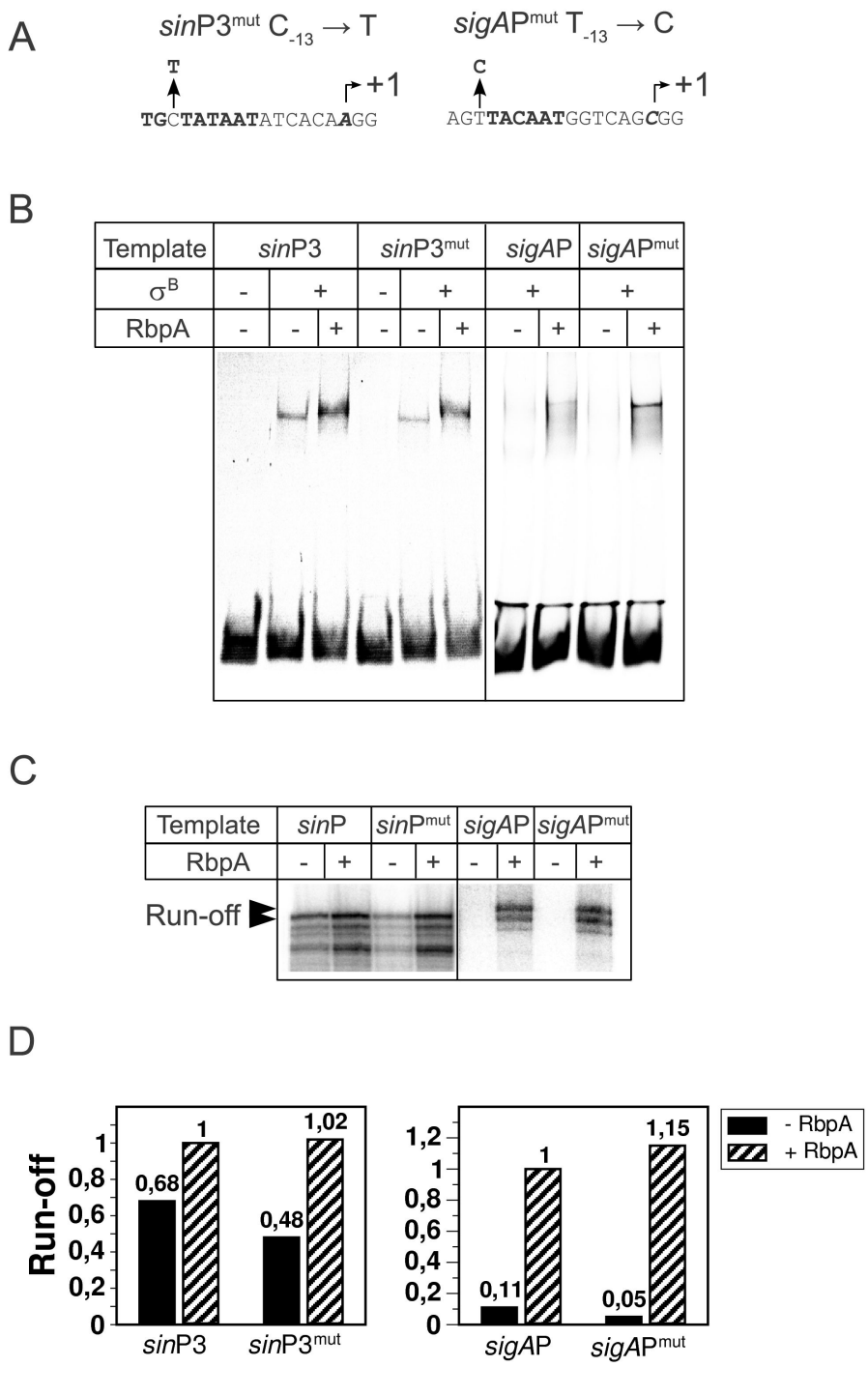
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Figure S4