Supplementary Data

for the manuscript

Bacillus subtilis RarA modulates replication restart

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This supplementary material contains:

Supplementary Tables (S1 and S2)

Supplementary Figures (S1 to S6)

SUPPLEMENTARY TABLES

Table S1. DNA substrates used in EMSA assays

Oligonucleotide	Sequence	Length
F1	TATAAAAAAAGCCAAGCTTGCATGCCTGCAGGTCGACT CTAGAGGATCCCCGGGTACCGAGCTCGAATTCACTGGC CGTCGTTTTACAACG	91-mer
F2	AATTCGTTGTAAAACGACGGCCAGTGAATTCGAGCTCG GTACCCGCCAGCCACAGTCAGACACACACACACTACAC ACAAAAAAAAAAA	89-mer
F3	TTTTTTTTTTTGTGTGTAGTGTGTGTGTGTCTGACTGTGG CTGG	44-mer
100U	GGGCGAATTGGGCCCGACGTCGCATGCTCCTCTAGACT CGAGGAATTCGGTACCCCGGGTTCGAAATCGATAAGCT TACAGTCTCCATTTAAAGGACAAG	100-mer
100D	CTTGTCCTTTAAATGGAGACTGTAAGCTTATCGATTTCG AACCCGGGGTACCGAATTCCTCGAGTCTAGAGGAGCAT GCGACGTCGGGCCCAATTCGCCC	100-mer
DNA substrate	**Description**	**Composition**[a]
fork[b]	Mimics a stalled fork with gaps in both strands	F1+F2
replicated fork[b]	Mimics a stalled fork with a gap in the lagging strand	F1+F2+F3
dsDNA	A 100 bp dsDNA	100U+100D
ssDNA[c]	A 91 nt ssDNA	F1

[a]DNA substrates were obtained by annealing the stated oligonucleotides having one of them [γ-^{32}P]-radiolabelled. Equimolar amounts of each oligonucleotide were heated in 50 mM phosphate buffer pH 7.0 to 95 ºC for 5 min, and annealing was performed cooling down to room temperature over 120 min. [b]The fork and replicated fork contain tailed 41-bp duplex DNA. [c]The 91-nt ssDNA can form secondary structures, with a predicted minimal free energy of -21.90 kcal/mol

Table S2. Rates of RarA catalyzed ATP hydrolysis in the presence of different effectors

Proteins[a]	DNA effector	k_{cat} min^{-1}
RarA (12.5 to 50 nM)	No DNA	<6
RarA (12.5 nM, 1 RarA/1600-nt)	+ ssDNA (20 μM)	170.2 ± 8.8
RarA (25 nM, 1 RarA/800-nt)	+ ssDNA (20 μM)	200.1 ± 2.4
RarA (50 nM, 1 RarA/400-nt)	+ ssDNA (20 μM)	213.8 ± 3.0
RarA (100 nM, 1 RarA/200-nt)	+ ssDNA (20 μM)	172.3 ± 0.5
RarA (200 nM, 1 RarA/100-nt)	+ ssDNA (20 μM)	102.5 ± 4.3
RarA (25 nM, 1 RarA/300-nt)	+ ssDNA (7.5 μM)	66.6 ± 3.2
RarA (25 nM, 1 RarA/400-nt)	+ ssDNA (10 μM)	100.2 ± 4.2
RarA (25 nM, 1 RarA/600-nt)	+ ssDNA (15 μM)	133.6 ± 2.2
RarA (25 nM, 1 RarA/800-nt)	+ ssDNA (20 μM)	174.6 ± 2.8
RarA (25 nM, 1 RarA/600-nt)	+ polydT DNA (15 μM)	7.0 ± 0.3
RarA (25 nM, 1 RarA/1200-nt)	+ polydT DNA (30 μM)	7.8 ± 0.2
RarA (25 nM, 1 RarA/600-bp)	+ cdsDNA (15 μM)	33.3 ± 1.3
RarA (25 nM, 1 RarA/300-bp)	+ 3′-ldsDNA (7.5 μM)	36.4 ± 3.2
RarA (25 nM, 1 RarA/600-bp)	+ 3′-ldsDNA (15 μM)	37.2 ± 3.2
RarA (25 nM, 1 RarA/300-bp)	+ 5′-ldsDNA (7.5 μM)	50.8 ± 1.2
RarA (25 nM, 1 RarA/600-bp)	+ 5′-ldsDNA (15 μM)	85.9 ± 1.9
RarA (25 nM, 1 RarA/300-bp)	+ bl-dsDNA (7.5 μM)	92.2 ± 3.7
RarA (25 nM, 1 RarA/600-bp)	+ bl-dsDNA (15 μM)	117.0 ± 10.3
RarA (25 nM, 1 RarA/300-bp)	+ dsDNA-ends (7.5 μM)	257.2 ± 12.3
RarA (25 nM, 1 RarA/600-bp)	+ dsDNA-ends (15 μM)	308.6 ± 8.8
RarA (25 nM, 1 RarA/600-nt)	+ ssDNA (15 μM)	133.3 ± 3.2
RarAK51A (50 nM, 1 RarA/300-nt)	+ ssDNA (15 μM)	<4
RarA (25 nM) + SsbA (18 nM)	No DNA	6.4 ± 0.9
RarA (25 nM) + SsbA (37 nM)	No DNA	6.7 ± 1.6
RarA (25 nM) + SsbA (18 nM)	+ polydT DNA (15 μM)	6.6 ± 0.8
RarA (25 nM) + SsbA (37nM)	+ polydT DNA (15 μM)	8.4 ± 0.5
RarA (25 nM) + SsbA (18nM)	+ 3′-ldsDNA (15 μM)	97.7 ± 10.2
RarA (25 nM) + SsbA (37nM)	+ 3′-ldsDNA (15 μM)	107.2 ± 12
RarA (25 nM) + SsbA (18nM)	+ 5′-ldsDNA (15 μM)	40.2 ± 3.3
RarA (25 nM) + SsbA (37nM)	+ 5′-ldsDNA (15 μM)	38.3 ± 3.7
RarA (25 nM) + SsbA (9 nM)	+ ssDNA (15 μM)	169.2± 4.3
RarA (25 nM) + SsbA (18 nM)	+ ssDNA (15 μM)	200.0 ± 3.4
RarA (25 nM) + SsbA (37 nM)	+ ssDNA (15 μM)	267.9± 4.5
RarA (25 nM) + SsbA (75 nM)	+ ssDNA (15 μM)	246.5 ± 2.3
RarA (25 nM) + SsbB (18 nM)	+ ssDNA (15 μM)	134.6 ± 10.2
RarA (25 nM) + SsbB (37 nM)	+ ssDNA (15 μM)	143.8 ± 9.3
RarA (25 nM) + SsbB (75 nM)	+ ssDNA (15 μM)	110.4 ± 6.3
RarA (25 nM) + SsbBA (18 nM)	+ ssDNA (15 μM)	176.4 ± 4.3
RarA (25 nM) + SsbBA (37 nM)	+ ssDNA (15 μM)	214.3± 4.4
RarA (25 nM) + SsbBA (75 nM)	+ ssDNA (15 μM)	226.3± 5.5

[a]Rates of RarA-mediated ATP hydrolysis were measured in the presence of the indicated DNA substrate, 80-nt long polydT oliginucleotide [polydT DNA], and 3,199 pGEM-3zf(+) based: circular ssDNA [ssDNA], supercoiled circular dsDNA [cdsDNA], SmaI linearized blunted linear dsDNA [bl-dsDNA], KpnI linearized 3′-tailed linear dsDNA [3′-ldsDNA], EcoRI linearized 5′-tailed linear dsDNA [5′-ldsDNA] and AluI-digested to obtain multiple dsDNA ends [dsDNA-ends]. The steady state kinetic parameters were derived from more than three independent experiments as the ones presented in Fig. 1 and Fig. S1; results are shown as mean ± SEM.

SUPPLEMENTARY FIGURES

Supplementary Figure S1

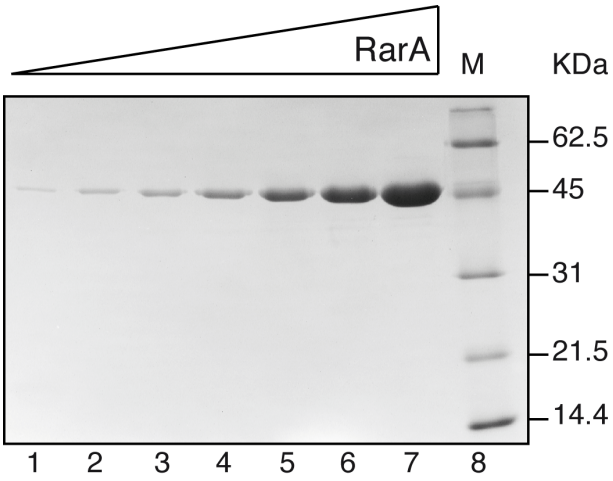

Figure S1. Purified *B. subtilis* RaA protein. Increasing concentrations (from 0.1 to 6.2 μg) of purified RarA (46.3 kDa, lanes 1-7) are shown. The purified protein was fractionated on a 12.5 % SDS-PAGE and gel was stained with Coomassie Brilliant Blue. The molecular masses (M) in kDa of the control proteins (lane 8) are indicated.

Supplementary Figure S2

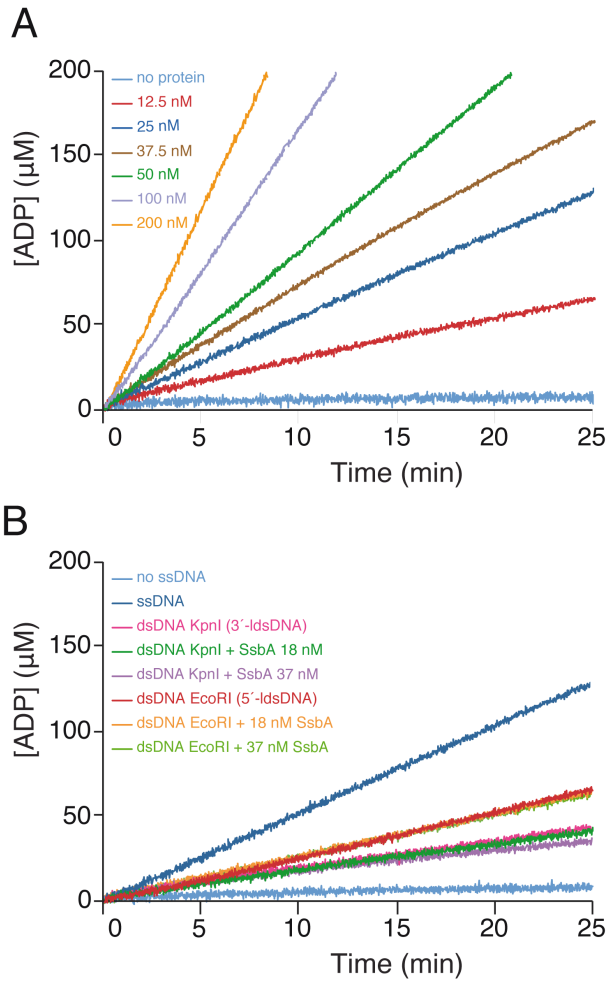

Figure S2. The RarA ATPase activity. (A) Circular 3199-nt ssDNA (20 µM) was incubated with increasing concentrations of RarA (12.5 to 200 nM) in buffer B containing 5 mM ATP, and the ATPase activity was measured (25 min, 37ºC). (B) The ATPase activity of RarA (25 nM) was measured in the presence of various duplex DNA substrates (EcoRI- [5´-ldsDNA] or KpnI-linearized [3´-ldsDNA] 3199-bp dsDNA, 15 µM) and two concentrations of SsbA (18 or 37 nM) as indicated. As a control, the ATP hydrolysis obtained with 25 nM RarA in the presence of circular ssDNA (ssDNA, 15 µM) is also depicted. The rate of ATP hydrolysed was calculated as described (see Methods). Representative graphics are shown and quantification of the results are expressed as the mean ± SEM of >3 independent experiments (see Table S2).

Supplementary Figure S3

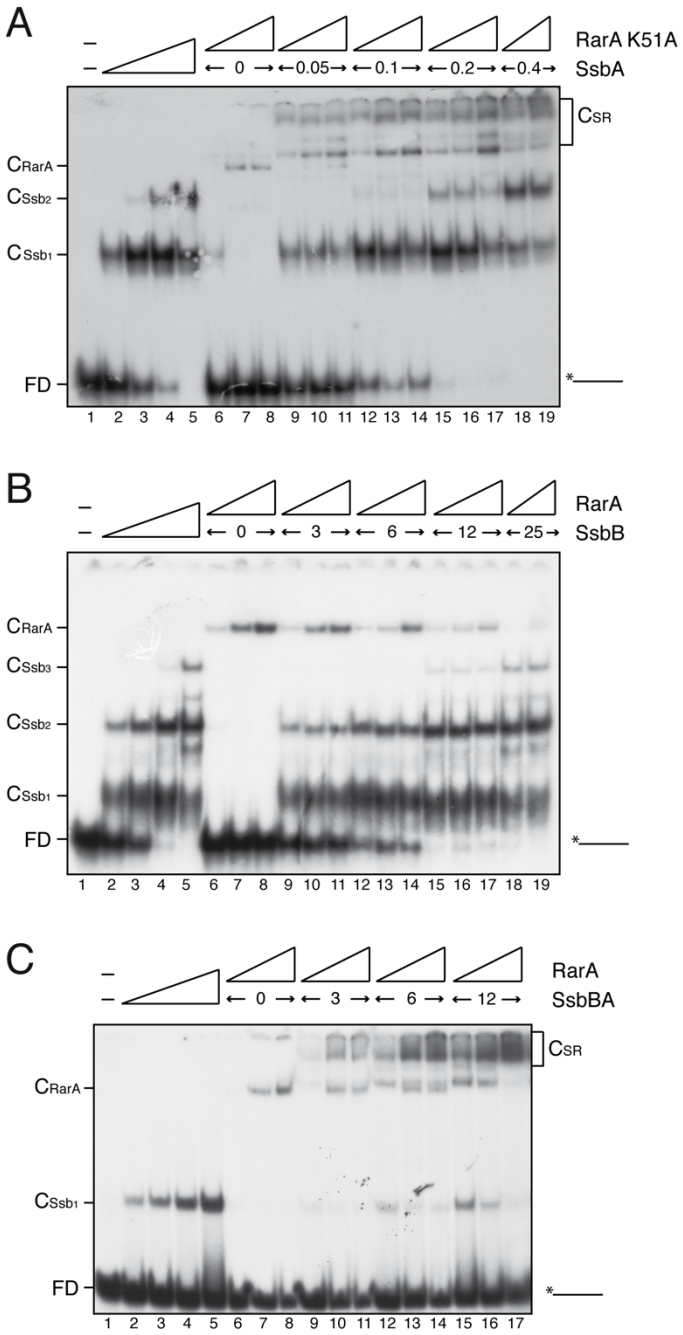

Figure S3. RarA binding to ssDNA is stimulated by the C-terminus of SsbA. (A) Cooperative binding of RarAK51A and SsbA to ssDNA. The indicated combinations of SsbA (0.05 to 0.4 nM) and RarAK51A (3 to 12 nM) were incubated with [γ^{32}P]-ssDNA (0.4 nM) in buffer B and protein-DNA complexes were separated by native electrophoresis. (B) and (C), The C-terminal region of SsbA is required for stimulation of RarA binding to DNA. In B, an EMSA assay showing the binding to [γ^{32}P]-ssDNA (0.5 nM) of the RarA protein (3 to 12 nM) in the presence of SsbB (3 tor 25 nM) which lacks the amphipathic C-terminal domain. In (C), RarA binding to the ssDNA substrate in the presence of the SsbBA chimera (3 tor 12 nM). Abbreviations: FD, free DNA; the different SSB-DNA complexes are indicated as C_{Ssb1} to C_{Ssb3}; C_{RarA}, RarA-DNA complex and C_{SR}, SsbA-DNA-RarA or SsbBA-DNA-RarA ternary complexes.

Supplementary Figure S4

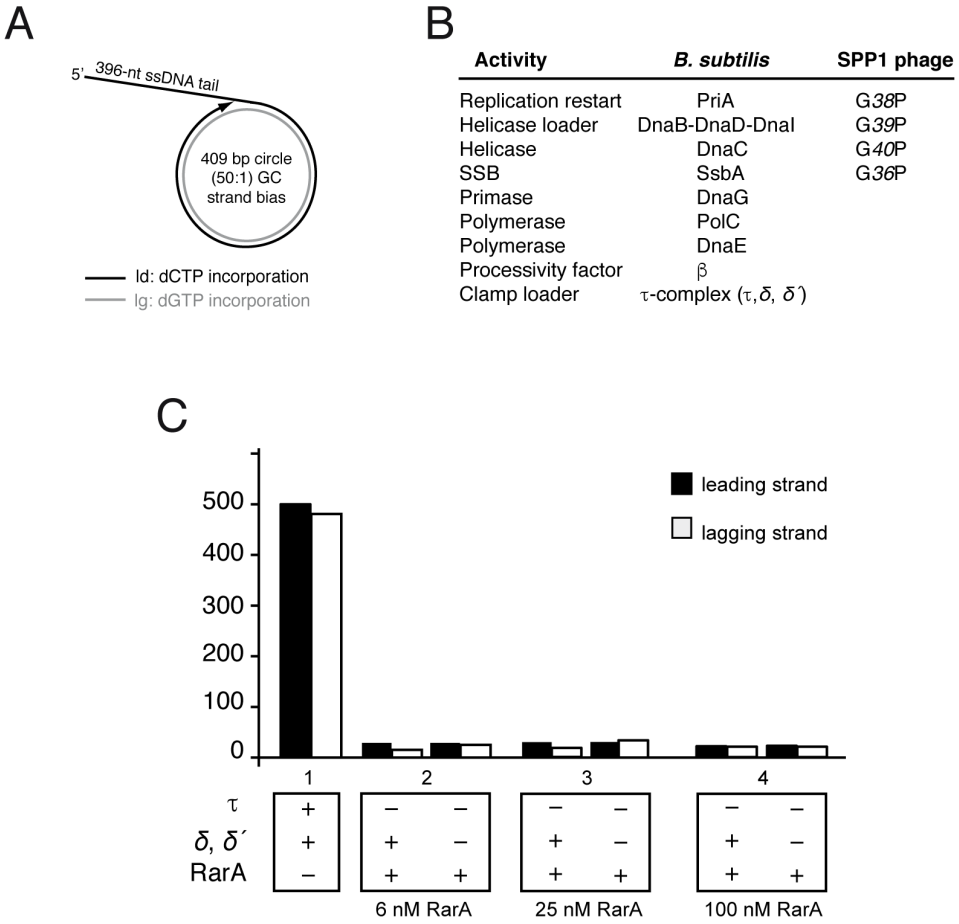

Figure S4. (A) Scheme of the synthetic nicked mini-circle substrate, which mimics a stalled replication fork, used in replication assays. Leading strand synthesis is primed by the pre-existing 3'-OH DNA end, and with this substrate, concatemeric dsDNA replication is obtained. (B) Activity and source of proteins used for *in vitro* DNA replication. (C) RarA does not act as an alternative clamp loader. Standard *in vitro* replication reactions were assembled with all *B. subtilis* replisome components except the clamp loader (composed by τδδ'), or only τ was substituted by increasing RarA concentrations (from 6 to 100 nM). DNA synthesis was quantified (15 min, 37ºC). As a control, the DNA synthesis obtained in a parallel complete reaction.

Supplementary Figure S5

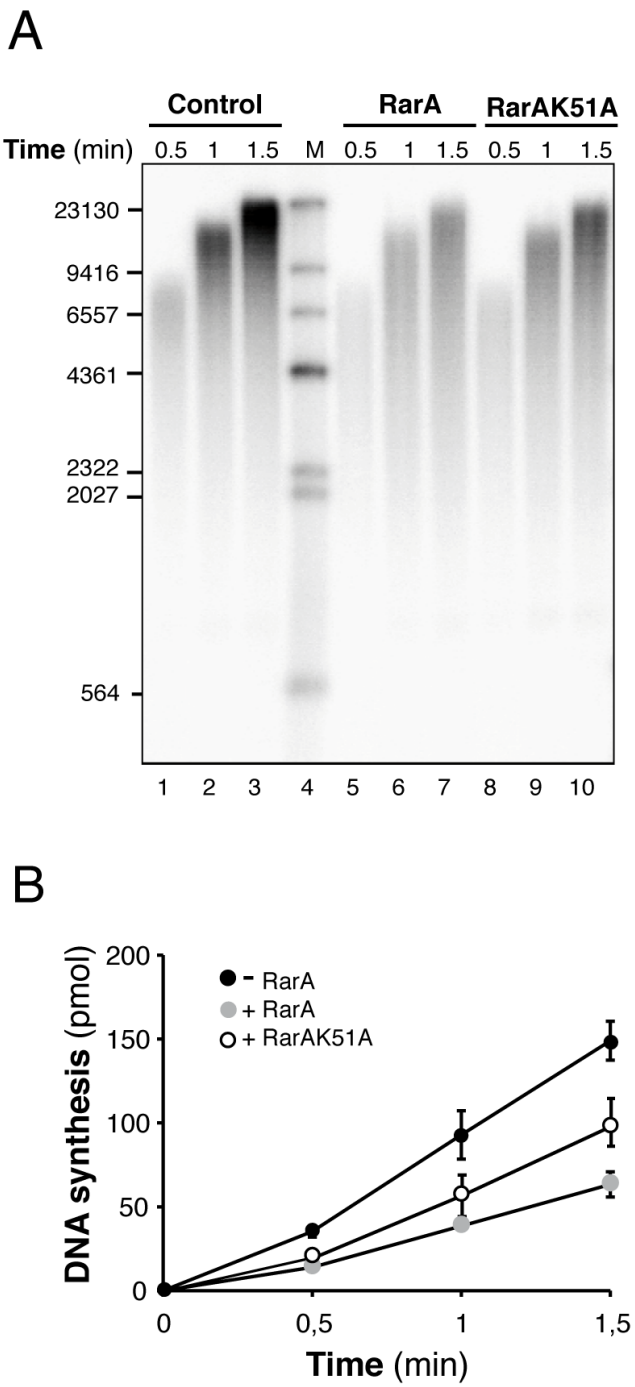

Figure S5. RarA and RarAK51A inhibit DNA replication. The *B. subtilis* replisome was assembled, in the absence or presence of 100 nM RarA or RarAK51A. After 5 min of incubation in presence of ATPγS, the *in vitro* DNA replication was started by dNTP (including [α-^{32}P]-dCTP) and ATP addition, and aliquots of the reactions were removed at the indicated times, and stopped as described in Material and Methods. (A) Alkaline agarose gel electrophoretic analysis of the leading products obtained after 0.5, 1, and 1.5 min in the presence of the RarA or RarAK51A. M, 3′-labelled *Hin*dIII-digested λ DNA. (B) Quantification of leading strand synthesis obtained. The results are expressed as the mean ± SEM of >3 independent experiments

Supplementary Figure S6

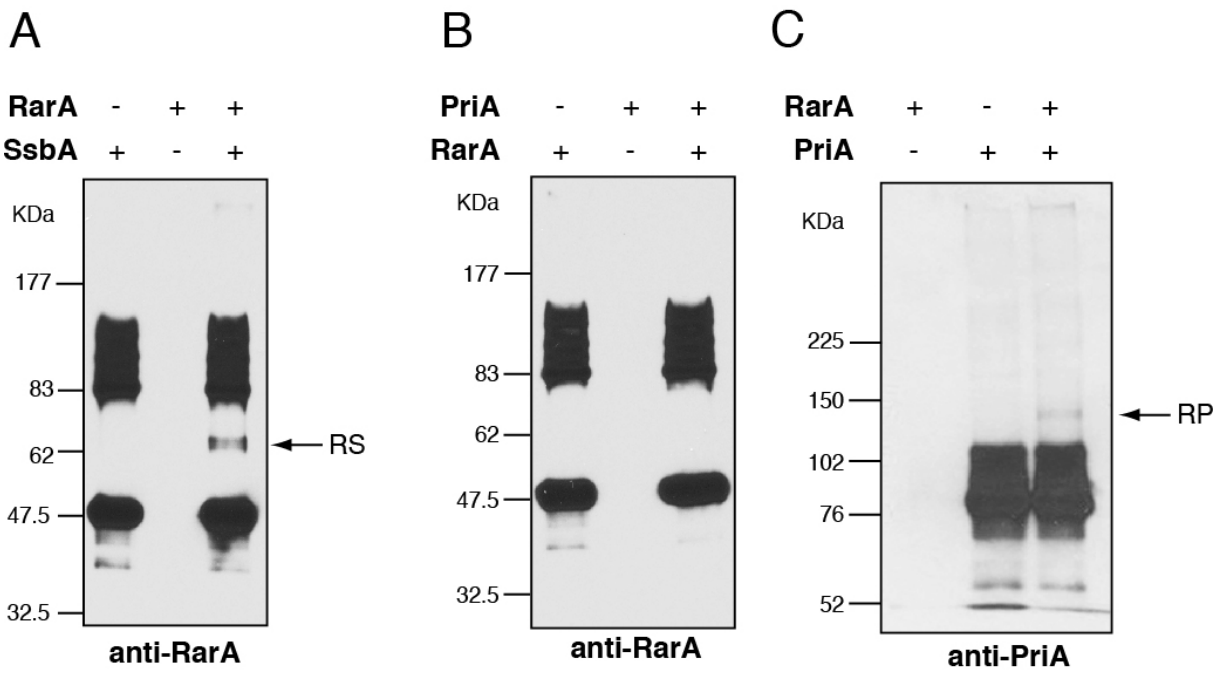

Figure S6. RarA may interact with SsbA and PriA. Protein cross-linking was performed incubating RarA with PriA or SsbA (1 μg each), in the presence of 50 μM DSS and buffer D (10 min, 37ºC). After SDS-PAGE, inmunoblots were developed using the polyclonal antibody indicated at the bottom of the gel. (A) Inmunoblot of cross-linked RarA and SsbA using anti-RarA polyclonal antibodies. The novel band of 75 kDa is indicated by RS. (B and C) Inmunoblot of cross-linked RarA and PriA developed using anti-RarA or anti-PriA polyclonal antibodies, respectively. The novel band of 141 kDa detected in (C) is indicated by RP. This band is masked in (B). Plus and minus denote the presence or absence of the indicated protein.