Guanosine tetraphosphate relieves the negative regulation of *Salmonella* pathogenicity island-2 gene transcription exerted by the AT-rich *ssrA* discriminator region

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Supplementary Information

Supplementary Experimental Procedures

NO_2^- determination. •NO synthesis by interferon-gamma (IFNγ)-stimulated J774A.1 cells was determined by measuring nitrite (NO_2^-) generated by the reaction of nitric oxide with oxygen. NO_2^- released into the culture supernatants by the macrophages 18 h after infection was measured after the addition of an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% N-1-naphthylethylenediamide hydrochloride in 2.5% phosphoric acid). The resulting change in color was read at 550 nm in a Versa Max spectrophotometer (Molecular Devices, Sunnyvale, CA). The NO_2^- concentration was determined from a standard curve prepared with $NaNO_2$.

Quantification of β-galactosidase expression. *Salmonella* strains expressing the *lacZY* translational fusions were lysed with chloroform in 980 μL of Z-buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$ and 0.05 M β-mercaptoethanol) containing 0.002% SDS (w/v). Samples were equilibrated at 30°C prior to the addition of 200 μL of 4 mg/mL ortho-nitrophenyl-β-galactoside (Sigma-Aldrich). The reactions were terminated by the addition of 500 μL of 1 M Na_2CO_3. β-galactosidase activity was measured with a Versa Max spectrophotometer

(Molecular Devices, Sunnyvale, CA) at 420 nm and 550 nm. Data are expressed in Miller units according to the equation:

(2) $1{,}000 \times [(OD_{420} - 1.75 \times OD_{550})]/(T(min) \times V(ml) \times OD_{600})$.

Table S1.

Bacterial

Strains

Strain	Genotype	Source
S. Typhimurium strain 14028s	Wild-type	ATCC
E. coli DH5α	F-ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK^-, mK^+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	ATCC
AV07270	Δ*dksA*::cm	[1]
AV08140	Δ*relA*::FRT Δ*spoT*::FRT	[2]
AV00203	Wild-type *sifA*::*lacZY*-km	[3]
AV15023	Δ*dksA*::cm *sifA*::*lacZY*-km	This study
AV15024	Δ*relA*::FRT Δ*spoT*::FRT *sifA*::*lacZY*-km	This study
AV00204	Wild-type *srfJ*::*lacZY*-km	[3]
AV15005	Δ*dksA*::cm *srfJ*::*lacZY*-km	This study
AV15006	Δ*relA*::FRT Δ*spoT*::FRT *srfJ*::*lacZY*-km	This study
AV00205	Wild-type *sspH2*::*lacZY*-km	[3]
AV15008	Δ*dksA*::cm *sspH2*::*lacZY*-km	This study
AV15009	Δ*relA*::FRT Δ*spoT*::FRT *sspH2*::*lacZY*-km	This study
AV00207	Wild-type *spiC*::*lacZY*-km	[3]
AV15011	Δ*dksA*::cm *spiC*::*lacZY*-km	This study
AV15012	Δ*relA*::FRT Δ*spoT*::FRT *spiC*::*lacZY*-km	This study
AV00206	Wild-type *sseE*::*lacZY*-km	[3]
AV15017	Δ*dksA*::cm *sseE*::*lacZY*-km	This study
AV15018	Δ*relA*::FRT Δ*spoT*::FRT *sseE*::*lacZY*-km	This study
AV15013	Wild-type pQF50-*ssaG*	This study
AV15014	Δ*dksA*::cm pQF50-*ssaG*	This study
AV15015	Δ*relA*::FRT Δ*spoT*::FRT pQF50-*ssaG*	This study
AV15019	Wild-type pQF50-*sseA*	This study
AV15020	Δ*dksA*::cm pQF50-*sseA*	This study
AV15021	Δ*relA*::FRT Δ*spoT*::FRT pQF50-*sseA*	This study
AV11276	Wild-type *sifA*::*luc*	[4]
AV15027	Δ*dksA*::cm *sifA*::*luc*	This study
AV15028	Δ*relA* Δ*spoT sifA*::*luc*	This study

AV14025	Wild-type Str^R	This study	30
AV13150	Δ*dksA*::cm Str^R	This study	
AV13146	Δ*ssrB*::km Str^R	This study	
AV13149	Δ*ssrB*::km Δ*dksA*::cm	This study	
AV14025	Δ*relA*::FRT Δ*spoT*::cm Str^R	This study	
AV14043	Δ*relA*::FRT Δ*spoT*::cm Δ*ssrB*::km	This study	
AV11228	Wild-type *ssrB*-FLAG	This study	
AV15189	Δ*dksA*::cm *ssrB*-FLAG	This study	
AV15190	Δ*relA*::FRT Δ*spoT*::FRT *ssrB*-FLAG	This study	
AV15162	Δ*ssrAB::*FRT	This study	
AV07104	Wild-type *ssrB*-3xFLAG	[5]	
AV15202	$ssrA_{Dsc}$ *ssrB*-3xFLAG	This study	
AV10369	Δ*dksA*::FRT *put*::*dksA*	[6]	
AV16176	Δ*relA*::cm Δ*spoT*::km *put*::*spoT*::FRT	This study	
AV18094	Wild-type *Salmonella* with pWSK29	This study	
AV18096	Wild-type *Salmonella* with pWSK29-ssrB-3xFLAG	This study	

Table S2.

Plasmids

Plasmid	Relevant Genotype	Source
pQF50	*bla lacZ*	[7]
pQF50-*ssaG*	*bla* P*ssaG*(-276/+35) *lacZ*	This study
pQF50-*spiC*	*bla* P*spiC*(-195/+166) *lacZ*	This study
pGEX6P1	*bla* P*lacZ* GST	GE Healthcare
pIDTSmart amp	*bla*	IDT
pTIM	*bla* pIDTSmart amp *rrnB* & *rpoC* term	This study
pTIM-*ssrA*	*bla* pIDTSmart amp P*ssrA(-258/+1202)*	This study
pSK::cm	*bla* FRT *cat* FRT pUC *ori* f1 *lacZ*α	This study
pKD13::km	*bla* FRT *ahp* FRT *oriR6K*	[8]
pTP223	P*lac*-gam-bet-exo	[9]
pWSK29	*bla lacZ*α T7/T3 *ori* f1 pSC101ori	[10]
pWSK29-ssrB-3xFLAG	*ssrB-3xFLAG* (-352 - +1564)	This study

Table S3. Primers

Gene		Primer Sequence
pTIM-*ssrA*	F:	CGGAATTCCGCCAGCATGAATCCCTCCTC
	R:	GGGGTACCCCTTGTGCTGGTAAACGTGTGC
Δ*ssrAB*	F:	ACTTACAATTTGAAAAATTATTTATTAAATAAACTGTTACGTGTAGGCTG GAGCTGCTTCG
	R:	CGAAGCGACCACGTTGCGCCACTGGGCAAGCTGTTTTTTCTGCATTCC GGGGATCCGTCGAC
$ssrA_{Dsc}$	F:	CATCGCCATCTTATTAAAAAGTAATTG
	R:	CAATTACTTTTTAATAAGATGGCGATGTAGGCACATCGTAACAGTTTAT TTAATAAATAATTT
*ssrA*5	F:	GAATTCACATTTATTTCGACTATAC
*ssrA*3	R:	GCTGCCCTCGCGAAAATTAAG
*ssrA*4	R:	GACAAAAGTACGTAATGACAG
*ssrB*4	F:	GAATTCAGAGCTACAGGAGCAGGATC
orf242-1	R:	CTGCAGCGCCTATAGTGTGATAAC
orf242-2	F:	ACTAGTTAGATTTCTTCCCCTCATTC
orf242-3	R:	GAGCTCATCAAAGCGTACCGTGGCGCCA
cmP2	F:	CTGCAGCATGGTCCATATGAATATCC
	R:	ACTAGTGTGTAGGCTGGAGCTGCTTC
Δ*relA*::FRT Δ*spoT*::FRT *put*::*spoT*		
spoT pSK	F:	TAGGGCCCAGGTATAGCGCTTTAGTGAATAAAAACCG
	R:	GCCTCGAGCTAGTTTCGGTTACGGGTGA
put::*spoT*	F:	TAGCGATGGGAGAGAGGACACGTTAATTATTCCATTTTAAAGGTATA GCGCTTTAGTGAATAAAAACCG
	R:	TACTGCGGGTATTAATGCTGAAAACATCCATAACCCATTGGTGTAGG CTGGAGCTGCTTC
ssrB-3xFLAG	F:	GAATTCAGAGCTACAGGAGCAGGATC (underlined, EcoRI site)
	R:	CTGCAGCGCCTATAGTGTGATAAC (underlined, PstI site)

Table S4. Primers and probes for qPCR and *in vitro* transcription

Gene		Primer Sequence
ssrA[1]	F:	ATATTACGCACAACCTTGCAT
	R:	CCAGTGAGCGATGTAGTAACCA
	Probe:	AAGCCGACGTCATCAACACCA
ssrA[2]	F:	TCATCGACTGGGTTATATATGAAG
	R:	AGATTGAGCAAATTCATAATGCTT
	Probe:	CTTTGGCACTTGATCACTATCGC
ssrB	F:	AGCGGCATTGCAAACAGT
	R:	TACCAATCATGGGATCAGCG
	Probe:	ATCGGGAAGCTATCCTGGCTG
ssaG	F:	TCCCACATGGCGCACCAG
	R:	ATGATTCCACTAAGCATATCCTTGA
	Probe:	AAGCGCAATTTGCCTTACAGCAG

[1]Primer set and probe used for *ssrA* qPCR

[2]Primer set and probe used for *in vitro* transcription

Supplementary Figure Legends

Fig S1. Interactions of *Salmonella* with J774A.1 cells. The amount of nitrite (NO_2^-) generated by macrophages 18 h after *Salmonella* infection was quantified by the Griess reaction (A). J774A.1 cells were stimulated with 200 U/ml IFNγ 24 h prior to infection, or treated with 960 μM of the selective iNOS inhibitor N-iminoethyl-L-lysine (L-NIL) since the time of infection. The data represent the mean ± S.D. from at least 3 biological replicates. *** $p < 0.001$ as compared to untreated control (A). Transcriptional analysis of major SPI2 promoters fused to a promoterless *lacZY* reporter in *Salmonella* (B). Fold induction is the ratio of β-galactosidase enzymatic activity 3 h after culture of *Salmonella* in 8 μM $MgCl_2$ N9 medium over controls grown in 10 mM $MgCl_2$ N9 medium. The data are the mean ± S.D. from 3 biological replicates. *** $p < 0.001$ as compared to wild-type controls.

Fig S2. Competitive index of *Salmonella* strains. Competitive indices of *Salmonella* strains recovered livers of C57BL/6 mice 3 d after infection. Mice were inoculated i.p. with 10^2 (A) or 10^5 (B) CFU of the indicated *Salmonella* strains. No detectable (nd) CFU were isolated for the Δ*relA* Δ*spoT* strain under the experimental conditions used in panel A. Competitive index was determined by the equation $(\text{strain 1/strain 2})_{output}/(\text{strain 1/strain 2})_{input}$. Non-significant (ns), or *$p < 0.05$.

Fig. S3. SsrB protein expression in Δ*dksA Salmonella* complemented with a *dksA* allele. SsrB expression was determined by Western blotting in the indicated strains of *Salmonella*. Δ*dksA Salmonella* was complemented with the low copy plasmid pWSK29 containing the *dksA* gene. Two independent clones are shown for comparison.

Fig. S4. Map of the pTIM plasmid used for the *in vitro* transcription reactions. pTIM plasmid containing two multiple cloning sites (MCS) and two Rho-independent terminators (A). DNA containing MCS 1 and MCS 2 (blue) and *rrnB* and *rpoC* terminators (underlined) was inserted into pIDTSmart backbone by *in vitro* synthesis (B). The plasmids resulting from cloning gene promoters into MCS 1 were used as templates for *in vitro* transcription reactions.

Fig S5. Cloning strategy for the construction of *Salmonella ssrA*$_{Dsc}$. The *ssrAB* locus was was cloned into pBluescript SK(+) containing *ssrAB*, *orf242*, and a chloramphenicol resistant cassette, yielding pSK-*ssrAB*-3xFLAG::cm plasmid. Mutations in the discriminator region were introduced by subcloning the *ssrA* promoter with a reverse primer containing the discriminator mutations (*ssrA*$_{Dsc}$-R) and the *ssrA5*-F containing an *EcoR*I site. The resulting product generated the *ssrA*$_{Dsc}$-P1 fragment. The *ssrA*$_{Dsc}$-P1 promoter was stitched by PCR to the fragment *ssrA*$_{Dsc}$-P2 containing an *Nde*I site. The *ssrA*$_{Dsc}$ was reintroduced to pSK-*ssrAB*-3xFLAG::cm by digesting and ligating with *EcoR*I and *Nde*I sites. Western blot analysis of SsrB in wild-type and *ssrA*$_{Dsc}$ *Salmonella* grown in high and low Mg^{2+} media (B). DnaK was used as internal control. Ratio of SsrB signal / DnaK signal was calculated from densitometry in ImageJ.

Fig S6. Full size Western blots. Blots developed with an Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare and visualized with a Molecular Imager ChemiDoc XRS+ system (Bio-Rad). Panel A depicts the full blot of the cropped image shown in Fig 3C, and panel B depicts the full blot of the cropped image shown in Fig 4D.

References

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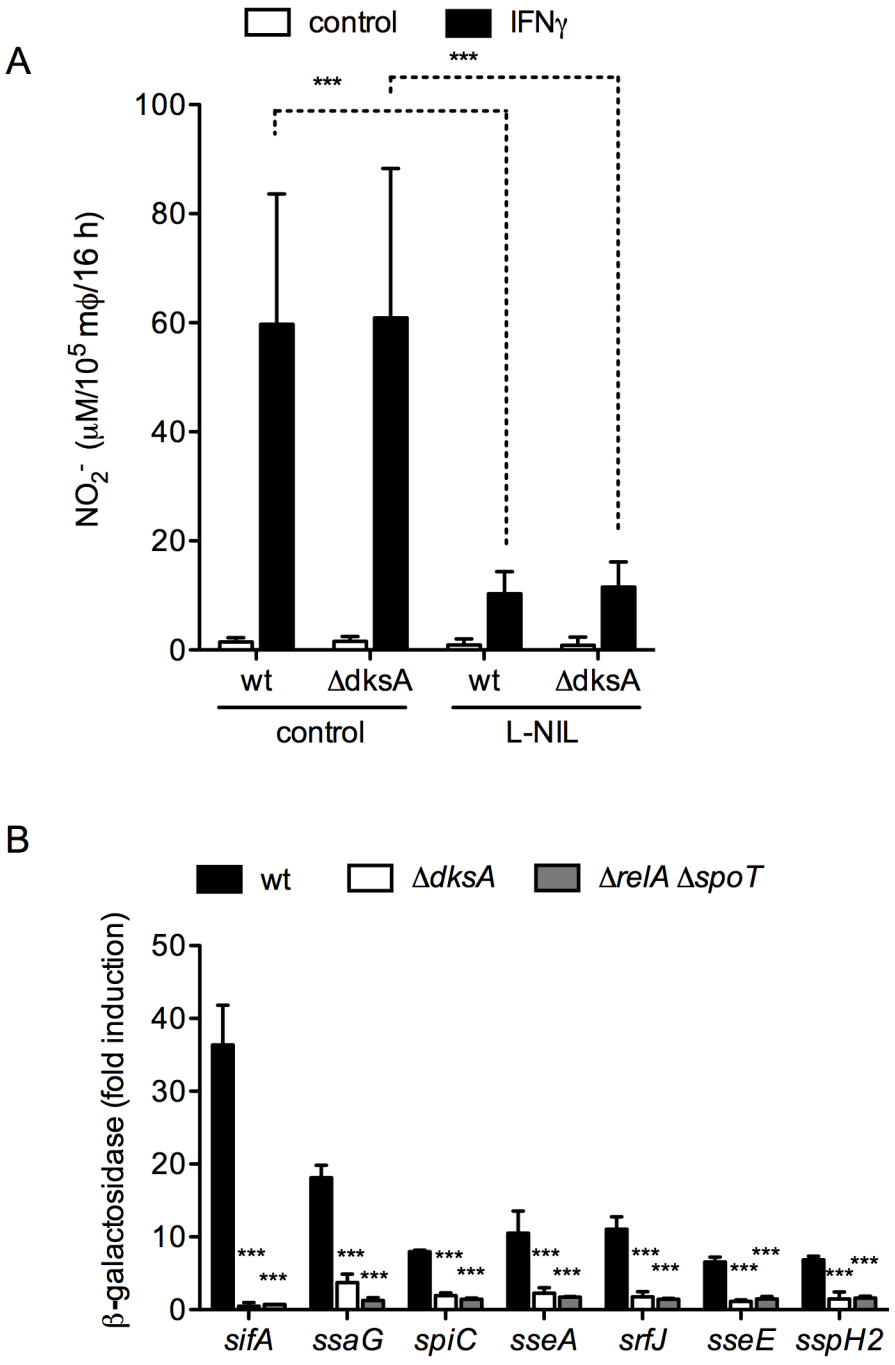
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IFNγ
***
***
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60
40
20
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wt
ΔdksA
wt
ΔdksA
control
L-NIL
B
wt
ΔdksA
ΔrelA ΔspoT
β-galactosidase (fold induction)
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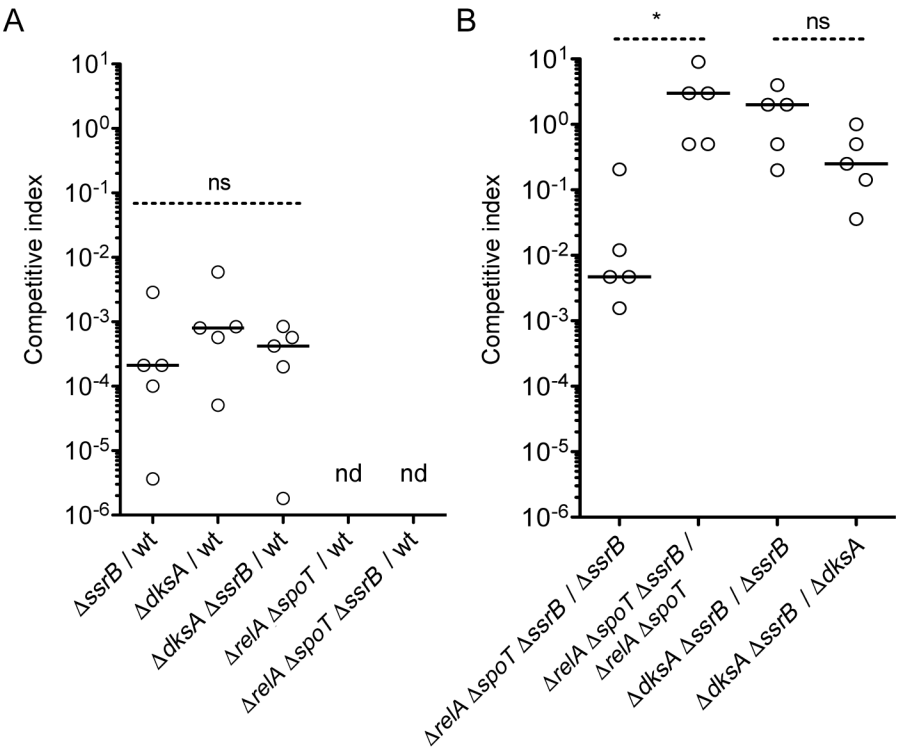
40
30
20
10
0
sifA
ssaG
spiC
sseA
srfJ
sseE
sspH2

Fig. S1


A
B
Competitive index
ns
nd
nd
*
ns
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ΔdksA / wt
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ΔrelA ΔspoT / wt
ΔrelA ΔspoT ΔssrB / wt
ΔrelA ΔspoT ΔssrB / ΔssrB
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ΔdksA ΔssrB / ΔdksA

Fig. S2

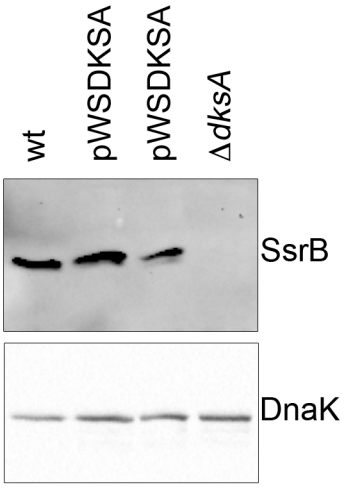
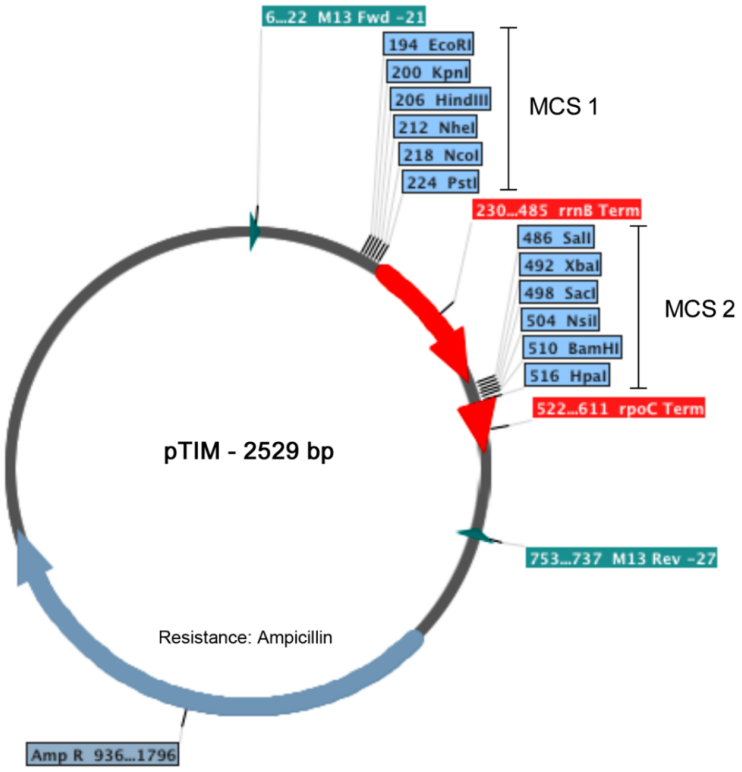


Fig. S3

A



B

MCS 1

>HindIII >PstI
>KpnI >NcoI
>EcoRI >NheI

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CTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGGGA

>*rrnB* Terminator

GCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAA

MCS 2

>SacI
>XbaI
>SalI >NsiI

GCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTCCTGTCgtcgacTCTAGAgagctcATGCATg

>HpaI
>BamHI

>*rpoC* Terminator

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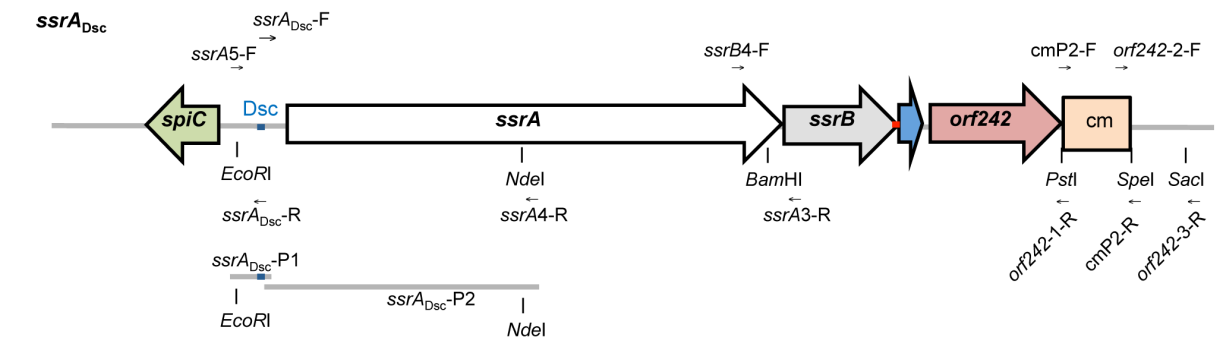
Fig. S4

A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orf242
cm
EcoRI
NdeI
BamHI
PstI
SpeI
SacI
ssrA_Dsc-R
ssrA4-R
ssrA3-R
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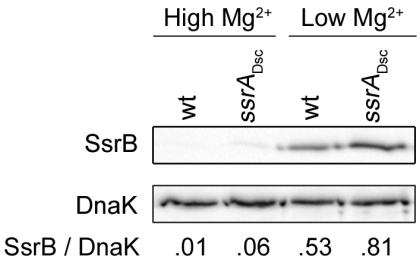
B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SsrB / DnaK
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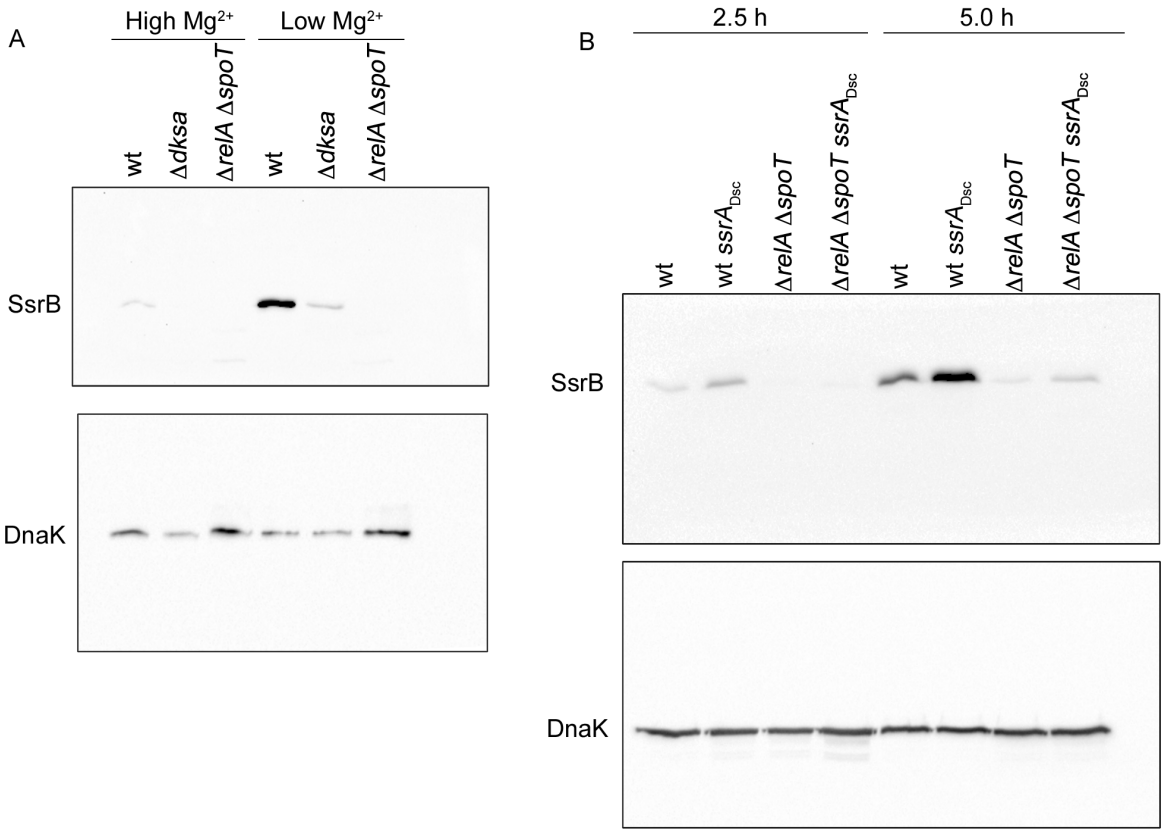
Fig. S5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B
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5.0 h
wt
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wt
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SsrB
DnaK

Fig. S6