Supplementary Information

Regulation of a muralytic enzyme-encoding gene by two non-coding RNAs

Renée J. St-Onge and Marie A. Elliot

Department of Biology and Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, Ontario, Canada

Supplementary Tables

Table S1. Bacterial strains, cosmids and plasmids

Strain/cosmid/ plasmid	Genotype, properties and/or use	Reference
***S. coelicolor* A3(2) strains**		
E120	M145 *rpfA-scr3097::aac(3)IV*	This study
M145	A3(2) SCP1$^-$ SCP2$^-$	Kieser, et al.[1]
***E. coli* strains**		
BW25113	Cosmid mutagenesis host; *lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner[2]
DH5α	Plasmid construction strain; F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Bethesda Research Laboratories
ET12567	Methylation-deficient strain; F$^-$ *dam13::*Tn*9 dcm6 hsdM hsdR recF143 zjj201::*Tn*10 galK2 galT22 ara14 lacY1 xyl5 leuB6 thi1 tonA31 rpsL136 hisG4 tsx78 mtli glnV44*	MacNeil, et al.[3]
***S. coelicolor* cosmids**		
StE41	*rpfA*$^+$ *scr3097*$^+$ *bla kan*	Redenbach, et al.[4]
StE41c	StE41 *rpfA-scr3097::aac(3)IV*	This study
Plasmids		
pFLUX	Integrative transcriptional reporter vector; *ori* (pUC18) *oriT* (RK2) *int* ϕBT1 *attP* ϕBT1 *luxCDABE* (promoterless) *aac(3)IV*	Craney, et al.[5]
pFLUX-Pos	P$_{ermE*}$ transcriptionally fused to *luxCDABE* in pFLUX	Sexton, et al.[6]
pIJ82	Integrative cloning vector; *ori* (pUC18) *oriT* (RK2) *int* ϕC31 *attP* ϕC31 *hyg*	Gift from H. Kieser
pIJ773	Plasmid carrying *oriT* (RK2) and *aac(3)IV* flanked by FLP recognition target sites for use in REDIRECT, *bla*	Gust, et al.[7]
pIJ790	λ RED recombination plasmid; *oriR101 repA101ts araC bet exo gam* P$_{araBAD}$ *cat*	Gust, et al.[7]
pMC222	*rpfA* 5′ UTR-*luxCDABE* under the transcriptional control of P$_{ermE*}$ in pFLUX (WT)	St-Onge, et al.[8]
pMC223	*rpfA* 5′ UTR Δ$_{37-91}$-*luxCDABE* under the transcriptional control of P$_{ermE*}$ in pFLUX (P2)[a]	St-Onge, et al.[8]
pMC224	*rpfA* 5′ UTR Δ$_{101-144}$-*luxCDABE* under the transcriptional control of P$_{ermE*}$ in pFLUX (P4)[a]	St-Onge, et al.[8]
pMC225	*rpfA* 5′ UTR Δ$_{151-175}$-*luxCDABE* under the transcriptional control of P$_{ermE*}$ in pFLUX (P6)[a]	St-Onge, et al.[8]
pMC226	*rpfA* 5′ UTR Δ$_{145-198}$-*luxCDABE* under the transcriptional control of P$_{ermE*}$ in pFLUX (P5-7)[a]	St-Onge, et al.[8]
pMC227	*rpfA* 5′ UTR Δ$_{92-214}$-*luxCDABE* under the transcriptional control of P$_{ermE*}$ in pFLUX (P3-7)[a]	St-Onge, et al.[8]
pMC230	*rpfA-flag*, fused to the 3′ end of the *rpfA* 5′ UTR, under the transcriptional control of P$_{ermE*}$; *ori* (pUC18) *oriT* (RK2) *int* ϕBT1 *attP* ϕBT1 *aac(3)IV*	St-Onge, et al.[8]
pMC231	*rpfA* 5′ UTR Δ$_{199-208}$-*luxCDABE* under the transcriptional control of P$_{ermE*}$ in pFLUX (LIN)[a]	St-Onge, et al.[8]

Table S1. Bacterial strains, cosmids and plasmids (continued)

Strain/cosmid/ plasmid	Genotype, properties and/or use	Reference
Plasmids		
pMC232	*rpfA* 5′ UTR (G14C G15C G16C C17G C18G C19G C20G)-*luxCDABE* under the transcriptional control of P_{ermE*} in pFLUX ($P0_{Prox.}$)[a]	This study
pMC233	*rpfA* 5′ UTR (G227C G228C G229C G230C A231T T232A C233G)-*luxCDABE* under the transcriptional control of P_{ermE*} in pFLUX ($P0_{Dist.}$)[a]	This study
pMC234	*rpfA* 5′ UTR (G14C G15C G16C C17G C18G C19G C20G G227C G228C G229C G230C A231T T232A C233G)-*luxCDABE* under the transcriptional control of P_{ermE*} in pFLUX ($P0_{Comp.}$)[a]	This study
pMC235	*rpfA* 5′ UTR (C27G G28C C29G A30T A31T C32G G33C C34G)-*luxCDABE* under the transcriptional control of P_{ermE*} in pFLUX ($P1_{Prox.}$)[a]	This study
pMC236	*rpfA* 5′ UTR (G217C C218G G219C T220A C221G G222C)-*luxCDABE* under the transcriptional control of P_{ermE*} in pFLUX ($P1_{Dist.}$)[a]	This study
pMC237	*rpfA* 5′ UTR (C27G G28C C29G A30T A31T C32G G33C C34G G217C C218G G219C T220A C221G G222C)-*luxCDABE* under the transcriptional control of P_{ermE*} in pFLUX ($P1_{Comp.}$)[a]	This study
pMC238	*rpfA* 5′ UTR (A55T C56G G57C G59C A60T)-*luxCDABE* under the transcriptional control of P_{ermE*} in pFLUX ($P2_{Prox.}$)[a]	This study
pMC239	*rpfA* 5′ UTR (T68A C69G C71G G72C T73A)-*luxCDABE* under the transcriptional control of P_{ermE*} in pFLUX ($P2_{Dist.}$)[a]	This study
pMC240	*rpfA* 5′ UTR (T239G T242A T244C T245G T246A T247C)-*luxCDABE* under the transcriptional control of P_{ermE*} in pFLUX [Poly(U)][a]	This study
pMC241	*rpfA* under the transcriptional control of P_{rpfA} (-504 to +816) in pIJ82[b]	This study
pMC242	*scr3097* under the transcriptional control of $P_{scr3097}$ (+457 to +1,046) in pIJ82[b]	This study
pMC243	*rpfA* and *scr3097* under the transcriptional control of P_{rpfA} and $P_{scr3097}$, respectively (-504 to +1,046), in pIJ82[b]	This study
pTRI-RNA 18S	Control template; Partial human 18S rRNA gene fragment under the transcriptional control of P_{T7}; *Eco*RI- and *Bam*HI-digested plasmid	Ambion
pUZ8002	Non-transmissible *oriT* mobilizing plasmid; RP4 *tra neo*	Paget, et al.[9]

[a] Numbering is relative to the *rpfA* transcriptional start site, as determined by 5′ rapid amplification of cDNA ends.[10]
[b] Numbering is relative to the *rpfA* start codon.

Table S2. List of PCR primers and probes

Primer/probe[a]	Sequence[b]	Restriction site	Reference
PCR primers			
5UTRF	GAA CAG TCG TCG CGT CAA G		St-Onge, et al.[8]
5UTRR	CAC TTA CCT TGG GTC CCC C		St-Onge, et al.[8]
DBLKOR	GGA CCG AGC CTA ACC GGC TCC GGG CGA TCG GCC AGC CGA TGT AGG CTG GAG CTG CTT C		This study
ermEF	GCA CTA GAT CTA GCC CGA CCC GAG CAC GCG C	*Bgl*II	St-Onge, et al.[8]
IVTrpfAUTR3′	GGA CGG ACG ACG GTG CTT GCC CTT GC		St-Onge, et al.[8]
IVTrpfAUTR5′	TAA TAC GAC TCA CTA TAG GGC GCT CGC CAT CGC GGG CCC		St-Onge, et al.[8]
P1dintF	***GCG TCG GTG ACC CCT AG***A CAT ATG CTG TTT TCC GGC A		This study
P1dintF2	***TCA CCT CGC AGC GCA GC***G TGA GGG GAT CAC ATA TGC T		This study
P1dintR	***CTA GGG G***TC ACC GAC GCC TGC GAG GTG		This study
P1dintR2	***GCT GCG*** CTG CGA GGT GAG CTG TCG GG		This study
P1pintF	***TAC CCA TGG CCC CGG GG***G ACA GCC GCA ACG CCG AAT C		This study
P1pintF2	***CCC GAC AGC GCG TTG CG***C GAA TCC TGC CAG CGG CCG T		This study
P1pintR	***CCC CGG G***GC CAT GGG TAC TGA TCG AA		This study
P1pintR2	***CGC AAC GC***G CTG TCG GGG CCC GCC ATG G		This study
P2dintF	***GGG AAC AGT CGA GGG CA***C AAG CGC CGA AGG CAG GAG C		This study
P2dintR	***TGC CCT*** CGA CTG TTC CCG TAC GGC CG		This study
P2pintF	***CCA GCG GCC GTT GCG CT***A CAG TCG TCG CGT CAA GCG C		This study
P2pintR	***AGC GCA*** ACG GCC GCT GGC AGG ATT CG		This study
pFLUXR	GCC GAA GTT GAT GGA CTG GA		This study
PLysCUTR	TAC GAC AAA TTG CAA AAA TAA TGT TGT CCT TTT AAA TAA GAT CTG ATA AAA TGT GAA CTC GCT CGC CGT CGC GGG CCC CGA CAG C		This study
PrpfAF-X	GCA CTT CTA GAC GAC AGT CTC TGG GCC ATC	*Xba*I	This study
PSCR3097-B	GCA CTG GAT CCC CTC GGG AGT CCA GGA TTT	*Bam*HI	This study
rpfAF	GAG TCC GGC GGC AAC TGG TC		Sexton, et al.[6]
rpfAR	GCT GGG ACT TGC TCG CCT GG		Sexton, et al.[6]
rpfAR2-K	GCA CTG GTA CCG ACG GCG ATG ACA CGG GTG G	*Kpn*I	St-Onge, et al.[8]
rpfAT-B	GCA CTG GAT CCC AAA GGC GGA ACA TGA AAA G	*Bam*HI	This study
rpoBF	TCG ACC ACT TCG GCA ACC GC		Hindra, et al.[11]
rpoBR	GCG CTC CAT ACG GGC GAG AC		Hindra, et al.[11]
SCO3096R-B2	GCA CTG GAT CCG GCA CGA GCA TCT CCT TCT T	*Bam*HI	This study
SCO3096R-X	GCA CTT CTA GAC CTC GGG AGT CCA GGA TTT	*Xba*I	This study
SCO3097F-B	GCA CTG GAT CCC GCT CCA CCG AGC AGA A	*Bam*HI	This study
SCO3097KOF	TTT GGA TCT CGT GAG AGA TAG GTC TCA GAA GCC GTG ATC ATT CCG GGG ATC CGT CGA CC		This study

Table S2. List of PCR primers and probes (continued)

Primer/probe[a]	Sequence[b]	Restriction site	Reference
PCR primers			
UintF	***ATC ACA TAG GCA GCG AC***C CGG CAA GGG CAA GCA CCG T		This study
UintR	***GTC GCT GCC*** TAT GTG ATC CCC TCA CCG AC		This study
Probes			
5S	CCC TGC AGT ACC ATC GGC GCT		Swiercz, et al.[12]
Scr3097-probe	TTC ATG TTC CGC CTT TGT TCC GTT C		This study

[a] All primers and probes were purchased from Integrated DNA Technologies.
[b] Sequences complementary to DNA flanking the region to be deleted are underlined once. Restriction sites are underlined twice. Promoter sequences are boxed. Primer extensions, incorporating specific mutations, are bold and italicized.

Table S3. PCR amplification conditions

PCR product	Frag-ment[e]	Primer[f]		Product length (bp)	Annealing temperature (°C)[g]	Extension time (min:sec)
		Forward	Reverse			
Extended knockout cassette[a]						
rpfA-scr3097	N/A	SCO3097KOF	DBLKOR	1,460	50-55	0:30
Confirming the mutant genotype[b]						
rpfA-scr3097	N/A	rpfAF	SCO3096R-B2	853	54	0:45
rpfA-scr3097:: aac(3)IV	N/A	SCO3097KOF	SCO3096R-B2	1,569	53	1:34
Cloning complementation and reporter constructs[c]						
P_{ermE*}-UTR[$P0_{Prox.}$]	5′	ermEF	P1pintR	309	59	0:15
P_{ermE*}-UTR[$P0_{Prox.}$]	3′	P1pintF	rpfAR2-K	308	N/A	0:15
P_{ermE*}-UTR[$P0_{Dist.}$]	5′	ermEF	P1dintR	522	N/A	0:15
P_{ermE*}-UTR[$P0_{Dist.}$]	3′	P1dintF	rpfAR2-K	95	65	0:15
P_{ermE*}-UTR[$P1_{Prox.}$]	5′	ermEF	P1pintR2	323	N/A	0:15
P_{ermE*}-UTR[$P1_{Prox.}$]	3′	P1pintF2	rpfAR2-K	294	N/A	0:15
P_{ermE*}-UTR[$P1_{Dist.}$]	5′	ermEF	P1dintR2	511	N/A	0:15
P_{ermE*}-UTR[$P1_{Dist.}$]	3′	P1dintF2	rpfAR2-K	106	61	0:15
P_{ermE*}-UTR[$P2_{Prox.}$]	5′	ermEF	P2pintR	349	N/A	0:15
P_{ermE*}-UTR[$P2_{Prox.}$]	3′	P2pintF	rpfAR2-K	268	N/A	0:15
P_{ermE*}-UTR[$P2_{Dist.}$]	5′	ermEF	P2dintR	362	N/A	0:15
P_{ermE*}-UTR[$P2_{Dist.}$]	3′	P2dintF	rpfAR2-K	255	N/A	0:15
P_{ermE*}-UTR[Poly(U)]	5′	ermEF	UintR	536	61	0:15
P_{ermE*}-UTR[Poly(U)]	3′	UintF	rpfAR2-K	81	N/A	0:15
SOE-PCR	N/A	ermEF	rpfAR2-K	600	62	0:15
rpfA	N/A	PrpfAF-X	rpfAT-B	1,342	65	0:20
scr3097	N/A	SCO3096R-X	SCO3097F-B	612	65	0:15
rpfA-scr3097	N/A	PrpfAF-X	PSCR3097-B	1,572	66	0:24
Confirming plasmid introduction[b]						
P_{ermE*}-UTR	N/A	ermEF	pFLUXR	715	61	0:45
rpfA	N/A	rpfAF	rpfAR	133	61	0:45
scr3097	N/A	SCO3096R-X	SCO3097F-B	612	60	0:45
rpfA-scr3097	N/A	PrpfAF-X	PSCR3097-B	1,572	53	1:34
RT-qPCR[d]						
rpfA CDS	N/A	rpfAF	rpfAR	133	61	0:45
rpfA 5′ UTR	N/A	5UTRF	5UTRF	54	57	0:45
rpoB	N/A	rpoBF	rpoBR	86	61	0:45

Table S3. PCR amplification conditions (continued)

PCR product	Frag-ment[e]	Primer[f]		Product length (bp)	Annealing temperature (°C)[g]	Extension time (min:sec)
		Forward	Reverse			
***In vitro* transcription templates[c]**						
In vitro transcription templates (P_{T7})	N/A	IVTrpfAUTR5′	IVTrpfAUTR3′	173-296	N/A	0:15
In vitro termination assay templates (P_{lysC})	N/A	PLysCUTR	pFLUXR	362-485	66	0:15

[a] PCR amplification of the extended apramycin resistance cassette was performed using Phusion High-Fidelity DNA polymerase. Amplification conditions were as follows: (i) an initial 3-min denaturation step at 98°C, (ii) 35 amplification cycles consisting of a 10-sec denaturation step at 98°C, a 30-sec annealing step at 50°C for the first 10 cycles then 55°C for the remaining 25 cycles, and a 30-sec extension step at 72°C, and (iii) a final 10-min extension step at 72°C.

[b] Colony PCRs used to confirm strain genotype were performed using *Taq* DNA polymerase with the following amplification conditions: (i) an initial 3-min denaturation step at 95°C, (ii) 35 amplification cycles consisting of a 30-sec denaturation step at 95°C, a 30-sec annealing step, and an extension step at 72°C, and (iii) a final 5-min extension step at 72°C.

[c] PCR amplifications of DNA for cloning experiments were performed using Phusion High-Fidelity DNA polymerase. Amplification conditions were as suggested by the manufacturer.

[d] qPCR amplifications of cDNA were performed using PerfeCTa SYBR Green SuperMix with the following amplification conditions: (i) an initial 3-min denaturation step at 95°C, (ii) 40 amplification cycles consisting of a 15-sec denaturation step at 95°C, a 45-sec annealing/extension step, and fluorescence measurement, and (iii) a melting step (65-95°C with 5-sec fluorescence reads every 0.5°C increase). PCR amplification efficiencies for each primer pair (mean ± standard deviation) were as follows: *rpfA* CDS = 1.076 ± 0.050; *rpfA* 5′ UTR = 1.087 ± 0.055; *rpoB* (for *rpfA* CDS assays) = 1.072 ± 0.038; *rpoB* (for *rpfA* 5′ UTR assays) = 1.087 ± 0.035.

[e] 5′, upstream fragment; 3′, downstream fragment.

[f] Primer sequences are located in Table S2.

[g] N/A, a two-step PCR amplification protocol was used.

Supplementary Figures

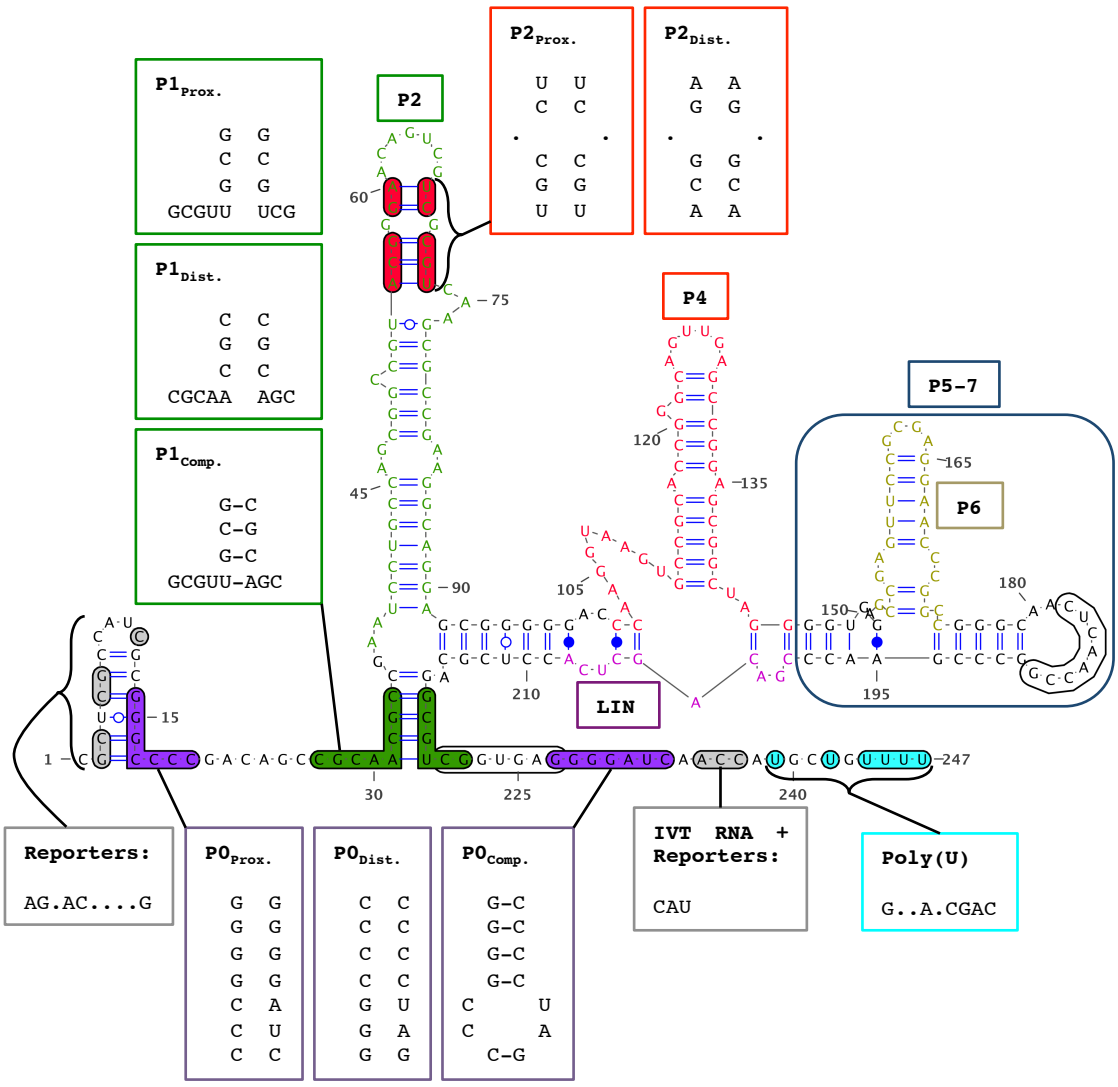

Fig. S1. Mutation of the *S. coelicolor rpfA* riboswitch. Regions of the *S. coelicolor rpfA* riboswitch were either deleted (colored letters) or substituted (highlighted letters). The 'P3-7' mutant, in which residues 92 to 214 were deleted, is not indicated here for clarity. Two G residues were added at the 5′ end of each RNA transcribed *in vitro* by phage T7 RNA polymerase. The 5′ end of the riboswitch in each transcriptional reporter construct carried additional mutations stemming from cloning. Both *in vitro* transcribed (IVT) RNA and reporter constructs carried an ACC → CAU mutation at the 3′ end of the riboswitch. Structure was drawn using VARNA.[13]

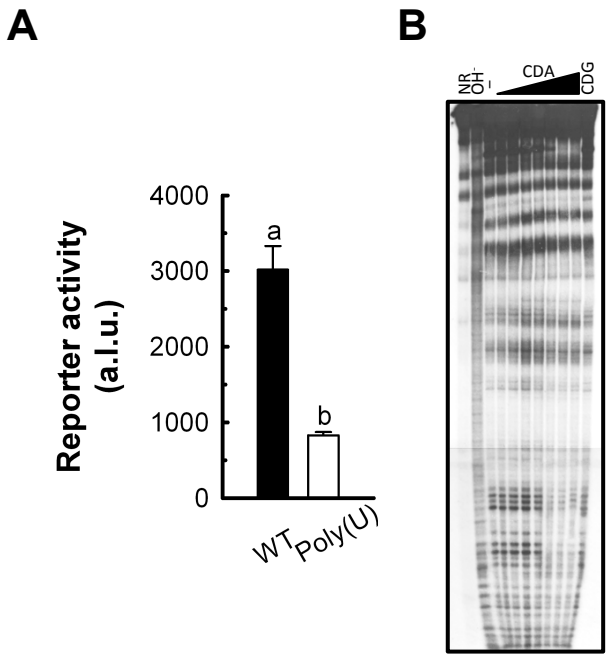

Fig. S2. Impact of the U-rich sequence on riboswitch activity and cyclic di-AMP binding. (A) *S. coelicolor* wild-type strains, constitutively transcribing the luciferase reporter gene cluster fused to the 3′ end of either the wild-type *rpfA* riboswitch (WT, M145/pMC222) or a mutant derivative in which the U-rich sequence was altered [Poly(U), M145/pMC240], were grown on a sporulation-conducive solid medium. Background-subtracted reporter activity was measured after 44 h. Data are expressed as mean ± standard error (n = 3). Group means annotated with different letters are significantly different. $t(4) = 6.94$, $p = 0.002$. Figure is representative of two independent experimental replicates. a.l.u., arbitrary luminescence units. (B) ^{32}P-5′-end-labeled poly(U) mutant *rpfA* riboswitch RNA was incubated in the absence of ligand (-) or in the presence of either cyclic di-AMP (CDA) or cyclic di-GMP (CDG) for ~40 h at room temperature. Cleaved products were size-fractionated on a denaturing urea-polyacrylamide gel and visualized by autoradiography. Image is representative of two independent replicates. NR, untreated RNA; OH^-, alkali-treated RNA.

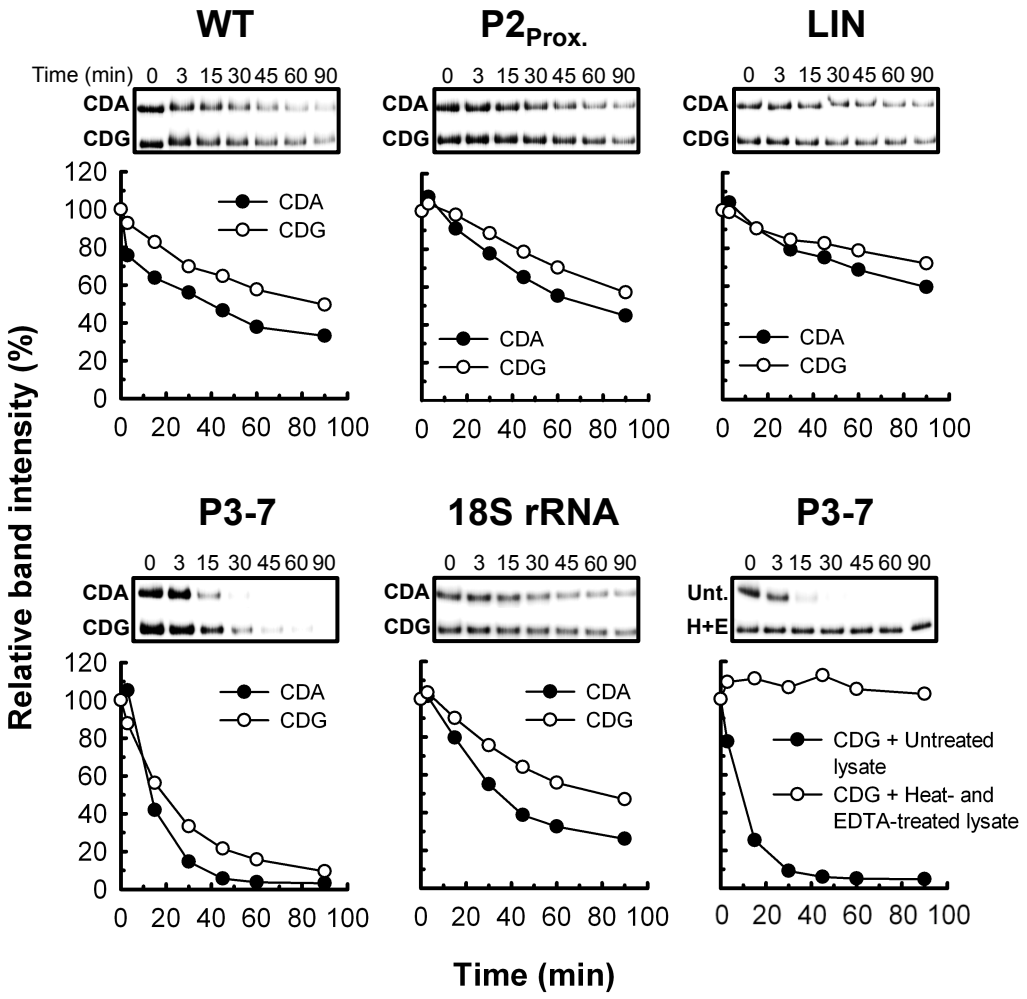

Fig. S3. Cyclic di-AMP impact on *rpfA* transcript stability. ^{32}P-5′-end-labeled *rpfA* riboswitch RNA and partial human 18S rRNA (negative control) were folded and incubated with cyclic di-AMP (CDA) or cyclic di-GMP (CDG) in a buffered solution. Degradation reactions were initiated by supplementing the mixtures with clarified wild-type *S. coelicolor* (M145) lysate. When appropriate, lysate was boiled with 20 mM EDTA at 95°C for 15 min (H+E). After the times indicated, an aliquot of the reaction was collected. Degradation products were size-fractionated on a denaturing urea-polyacrylamide gel, visualized by phosphorimaging, and full-length transcripts were quantified using densitometry. Transcript abundance at any given time-point was expressed relative to transcript levels at 0 min. Figure is representative of two independent experimental replicates. Full-length gel images are presented in Fig. S4. Unt., untreated lysate.

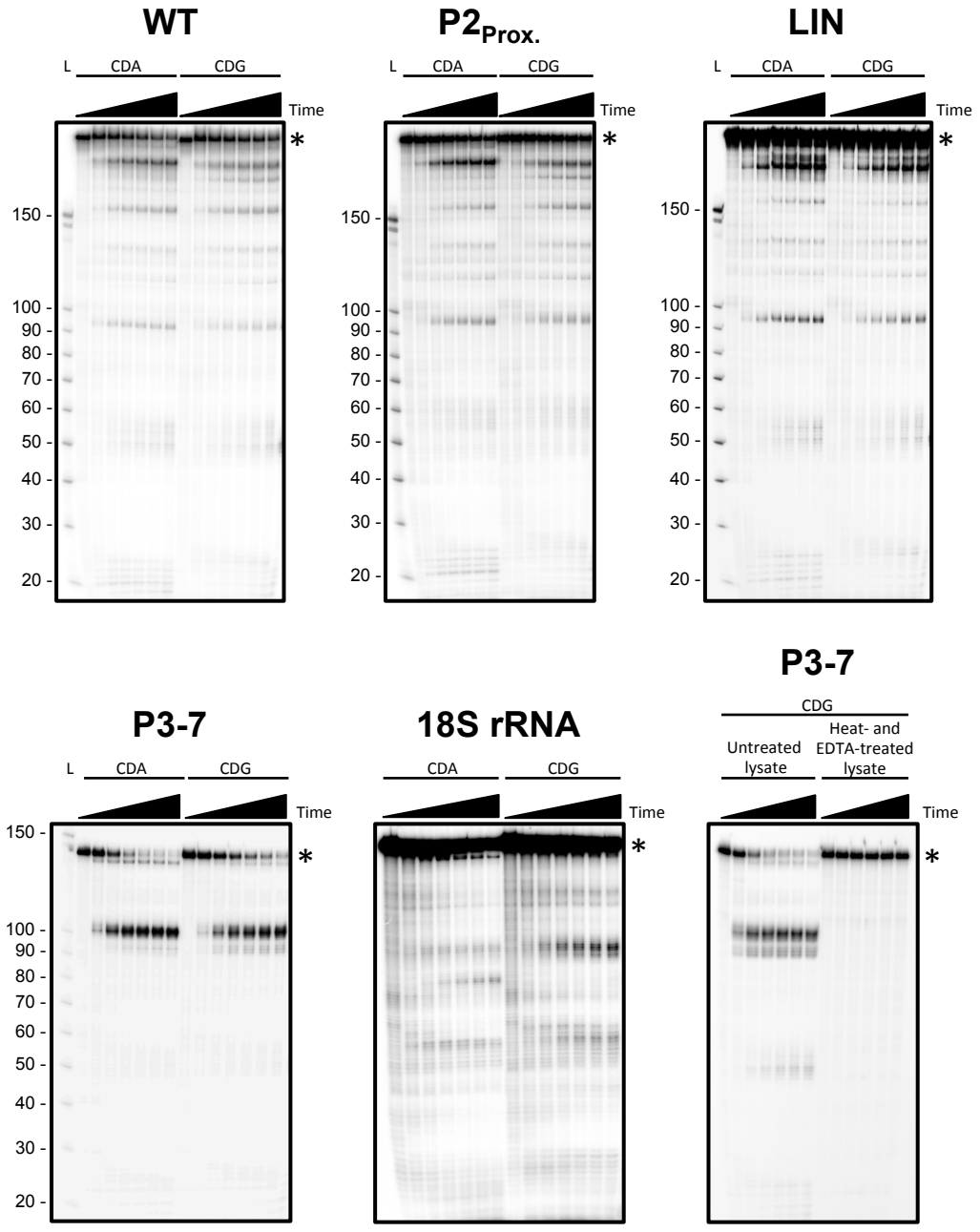

Fig. S4. Denaturing polyacrylamide gel electrophoresis analysis of riboswitch RNA degradation products. ^{32}P-5′-end-labeled *rpfA* riboswitch RNA and partial human 18S rRNA (negative control) were folded and incubated with cyclic di-AMP (CDA) or cyclic di-GMP (CDG) in a buffered solution. Degradation reactions were initiated by supplementing the mixtures with clarified wild-type *S. coelicolor* (M145) lysate. When appropriate, lysate was boiled with 20 mM EDTA at 95°C for 15 min. Aliquots of the reaction were collected periodically over the course of 90 min. Degradation products were size-fractionated on a denaturing urea-polyacrylamide gel and visualized by phosphorimaging. Figure is representative of two independent experimental replicates. L, Decade Marker RNA (Ambion), in nt; *, full-length transcript.

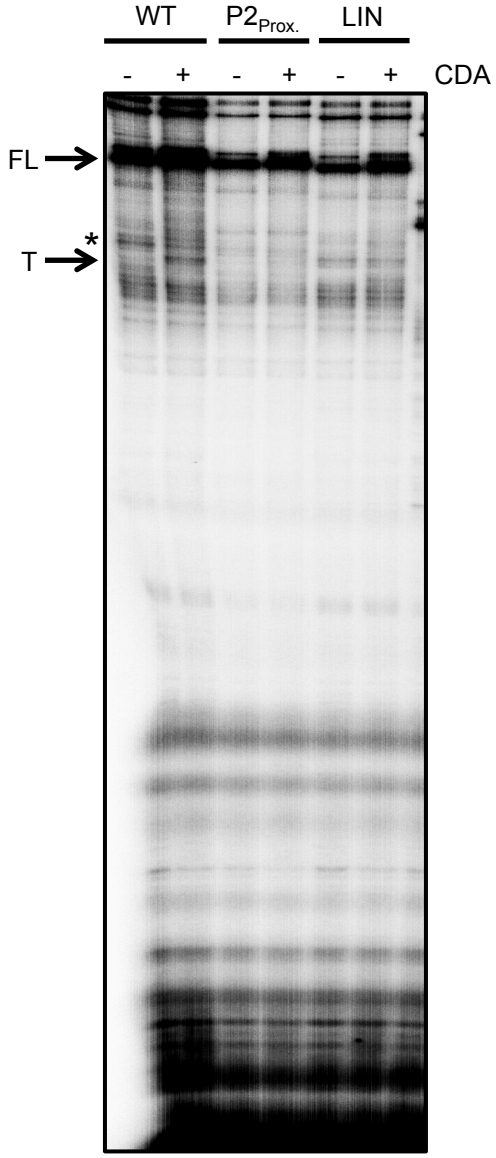

Fig. S5. Denaturing polyacrylamide gel electrophoresis analysis of RNA transcribed *in vitro* by bacterial RNA polymerase. Wild-type (WT) and mutant ($P2_{Prox.}$, LIN) *rpfA* riboswitches were transcribed *in vitro* from an engineered *B. subtilis lysC* promoter using *E. coli* σ^{70}-RNA polymerase. Reactions were supplemented with 0 µM (-) or 500 µM (+) cyclic di-AMP (CDA). Products were intrinsically labeled with ^{32}P, size-fractionated on a denaturing urea-polyacrylamide gel and visualized by phosphorimaging. Image is representative of three biological replicates. FL, full-length transcripts; *, End of UTR(WT)-*rpfA* moiety (39 nt into the *rpfA* coding sequence); T, terminated transcripts.

A

```
Streptomyces_scabiei_8722                          ----------------------AAAAGCCCCGCCCCGGTGCGTACGGGGGAATGCGCAC
Streptomyces_avermitilis_MA4680                    ------------CGGGACAAGAAAAAGCCCCCCCCCGGTGCGTACGGGGGAAGACGCAC
Streptomyces_hygroscopicus_subsp_jinggangensis_TL01 ----------------------AAAATCCCCGCCCCGGTGCGTACGGGGGAACACGCAC
Streptomyces_hygroscopicus_subsp_jinggangensis_5008 ----------------------AAAATCCCCGCCCCGGTGCGTACGGGGGAACACGCAC
Streptomyces_coelicolor_A32                        GAACGGAACAAAGGCGGAACATGAAAAGTCCCGCCCCGGTGCGTACGGGGGAACACGCAC
Streptomyces_davawensis_JCM4913                    ---------------------------CCCGCCCCGGTGCGTACGGGGGAACACGCAC
                                                                               ************************  *****

Streptomyces_scabiei_8722                          CGGGGCGGG----------
Streptomyces_avermitilis_MA4680                    CGGAGCGGG----------
Streptomyces_hygroscopicus_subsp_jinggangensis_TL01 CGGGGCGGG----------
Streptomyces_hygroscopicus_subsp_jinggangensis_5008 CGGGGCGGG----------
Streptomyces_coelicolor_A32                        CGGGGCGGGAGTCACGGGAC
Streptomyces_davawensis_JCM4913                    CGGGGCGGG----------
                                                   *** *****
```

B

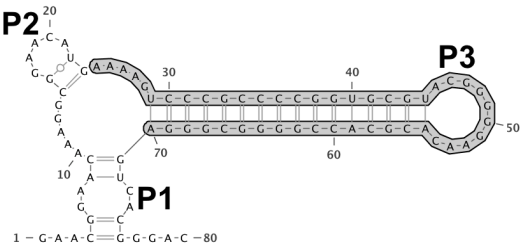

Fig. S6. Bioinformatic analysis of *scr3097* homologues from streptomycetes. (A) Bacterial genomes were searched for sequences similar to the *S. coelicolor scr3097*. Similar sequences were aligned. Asterisks indicate conserved nucleotides. (B) Predicted secondary structure of the *S. coelicolor* Scr3097. Conserved sequences and structures are highlighted in gray. The structure was drawn using VARNA.[13]

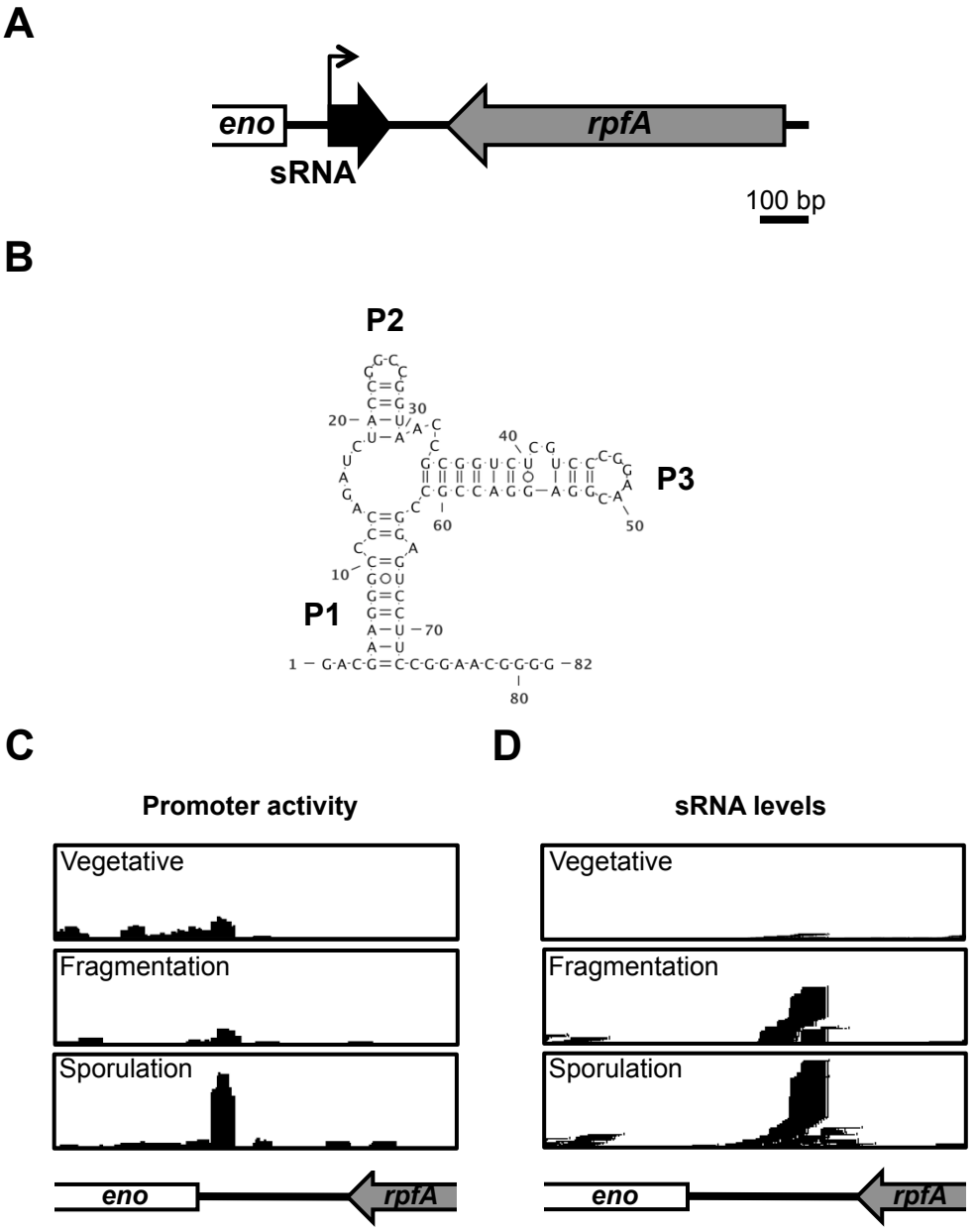

Fig. S7. The *rpfA*-associated sRNA of *S. venezuelae*. (A) Genetic context of the *rpfA*-associated sRNA gene of *S. venezuelae*. The black elbow arrow denotes the transcriptional start site of the sRNA gene as predicted using 5′ triphosphate end-capture RNA-seq (Matthew Bush and Mark Buttner, unpublished). Genes were drawn to scale. (B) Predicted secondary structure of the mature *S. venezuelae* sRNA. The structure was drawn using VARNA.[13] (C) *S. venezuelae* wild-type strain ATCC 10712 was grown at 30°C in MYM broth. Promoter activity downstream of *rpfA* was monitored throughout development using 5′ triphosphate end-capture RNA-seq (Matthew Bush and Mark Buttner, unpublished). (D) *S. venezuelae* wild-type strain ATCC 10712 was grown at 30°C in MYM broth. sRNA transcripts were quantified using RNA-seq (Emma Sherwood, unpublished). Relative read coverage is indicated.

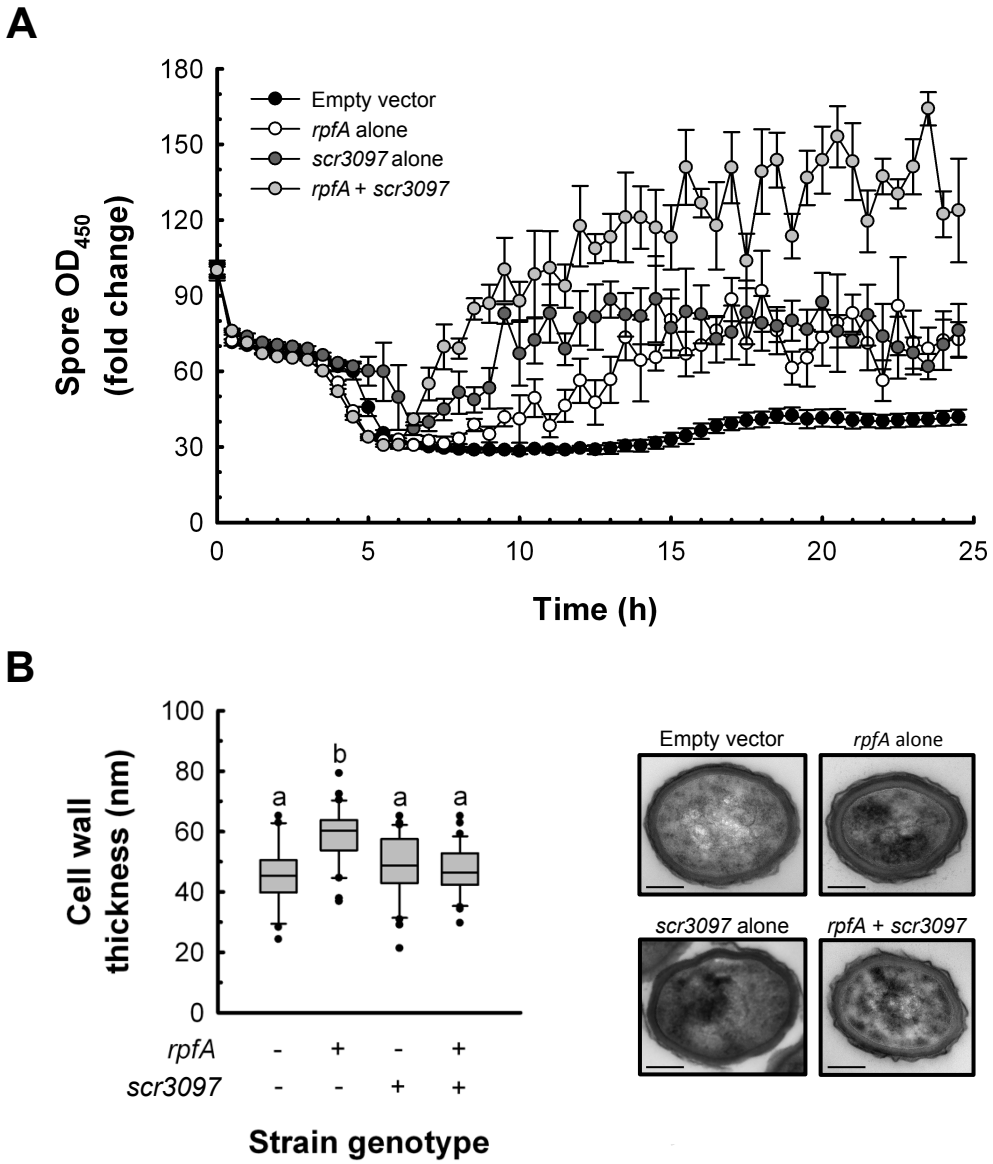

Fig. S8. Impact of *scr3097* on spore germination and sporulation. (A) Spores of *S. coelicolor rpfA-scr3097* deletion mutant, complemented with or without an ectopic copy of *rpfA* and/or *scr3097*, were germinated at 30°C in TSB:YEME (50:50). The background-subtracted OD_{450} was measured periodically and expressed as fold changes relative to initial values, at 0 h. Data are presented as mean ± standard error (n = 4). (B) The *S. coelicolor rpfA-scr3097* deletion mutant, complemented with or without an ectopic copy of *rpfA* and/or *scr3097*, was grown for 7 days at 30°C on MYM agar. Spores were visualized by transmission electron microscopy, and spore wall thickness was measured. *Left panel.* Box plots specify the median (center line), the 25th and 75th percentile (bottom and top of box, respectively), the 10th and 90th percentile (lower and higher whiskers, respectively), and the 5th and 95th percentile (lower and higher dots, respectively) (n = 29-32). Group means annotated with different letters are significantly different. $F(3,120) = 12.49$, $p < 0.001$. *Right panel.* Transmission electron micrographs. Bar, 200 nm. Empty vector, E120/pIJ82; *rpfA* alone, E120/pMC241; *scr3097* alone, E120/pMC242; *rpfA* + *scr3097*, E120/pMC243.

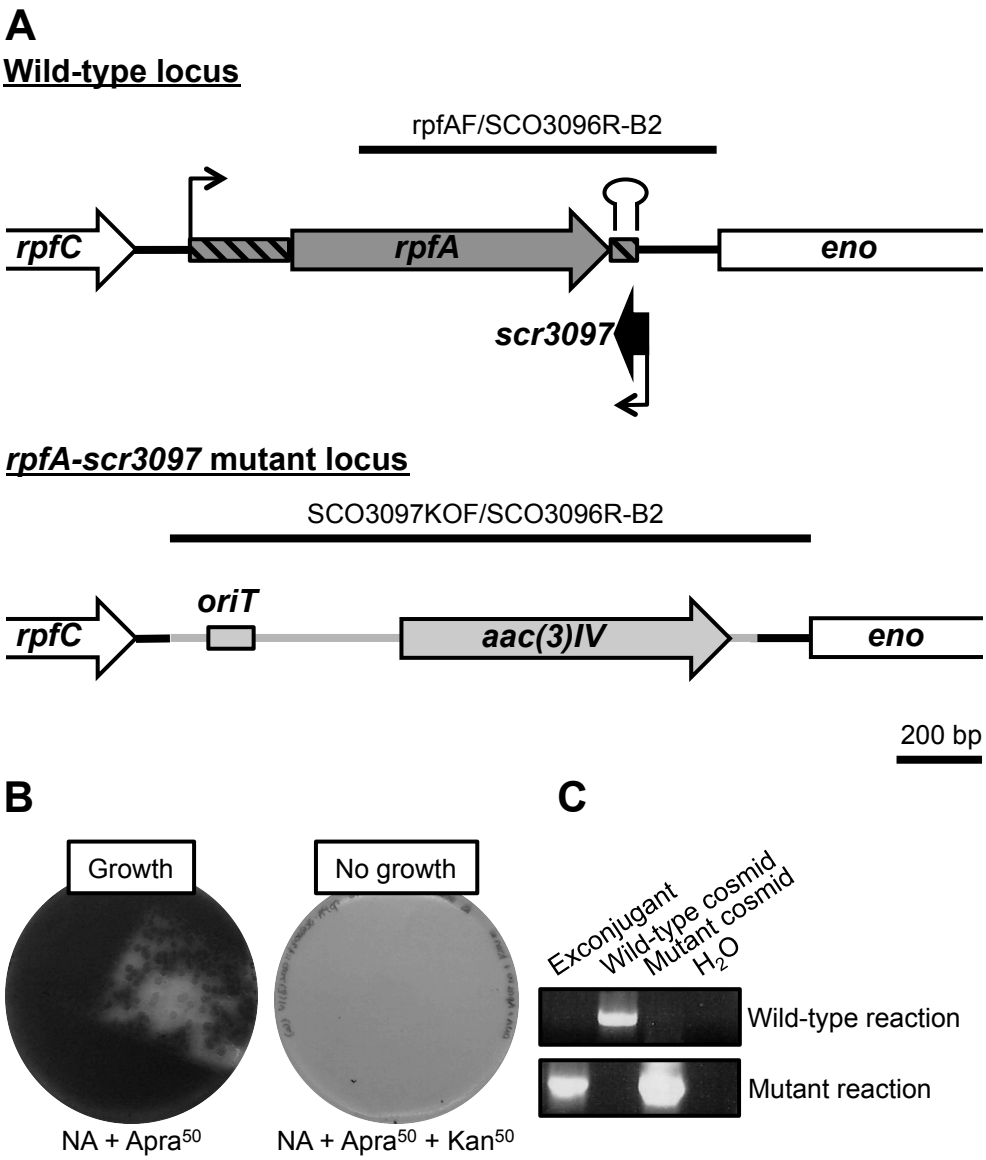

Fig. S9. Construction of the *rpfA-scr3097* deletion mutant. (A) Schematic of the *rpfA-scr3097* region in the wild-type and the deletion mutant. Hatched gray blocks indicate the location of the *rpfA* 5′ and 3′ UTRs, and black elbow arrows denote the transcriptional start sites of *rpfA* and *scr3097* as predicted using 5′ rapid amplification of cDNA ends[10] and RNA-seq,[14] respectively. Thick black lines above genes specify the location of the PCR products used to confirm the strain genotype. Primer pairs used are indicated above lines. Schematics were drawn to scale. (B) The *S. coelicolor rpfA-scr3097* deletion mutant (E120) was grown at 30°C on nutrient agar (NA) supplemented with 50 μg mL^{-1} apramycin ($Apra^{50}$) ± 50 μg mL^{-1} kanamycin (Kan^{50}). (C) The genotype of the *rpfA-scr3097* deletion mutant was confirmed by PCR-amplifying the wild-type and mutant loci from crude cell lysates. Wild-type (StE41) and mutant (StE41c) cosmids served as controls. An equal volume of water was added to the 'no template' negative control reactions (H_2O) instead of DNA template.

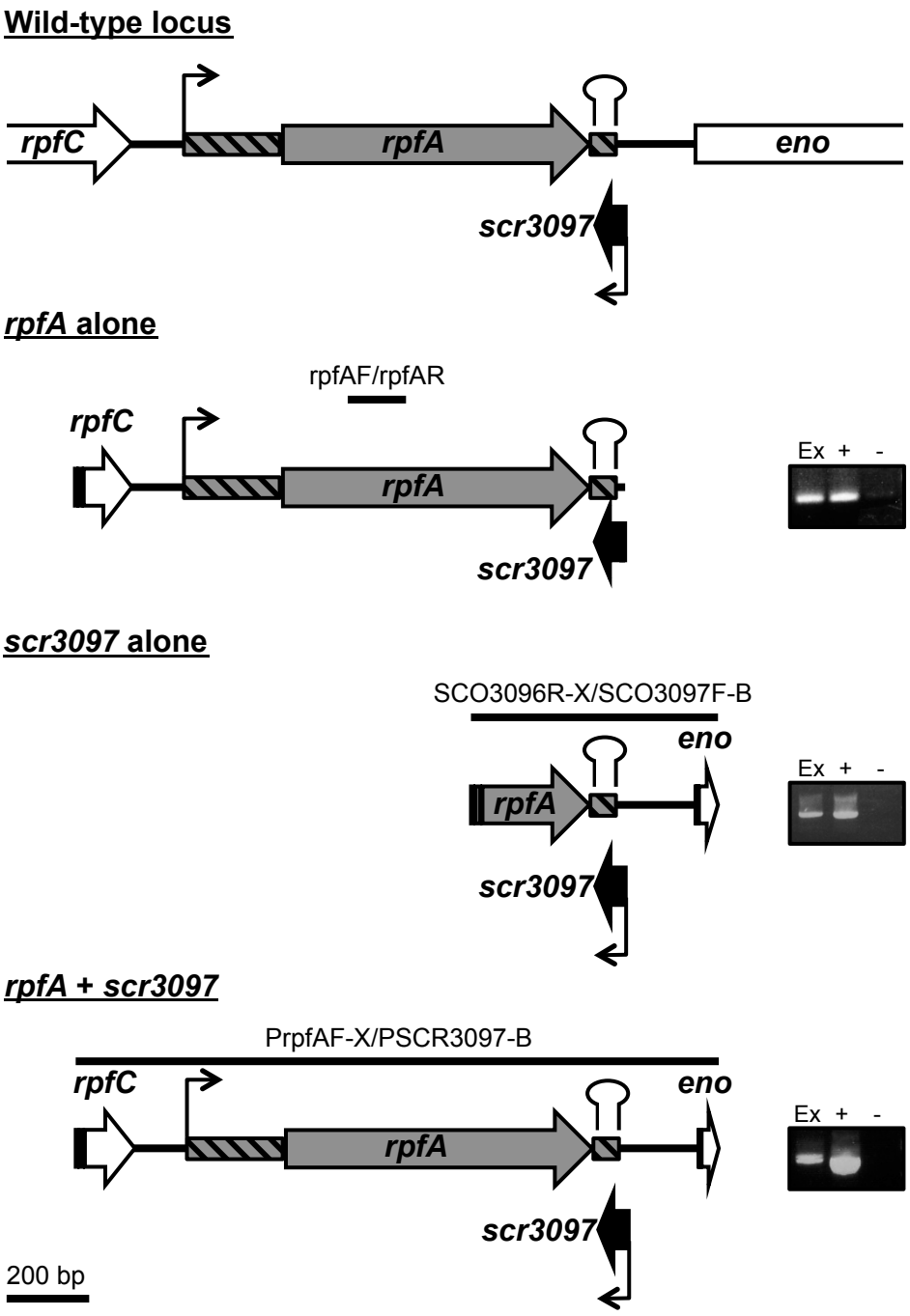

Fig. S10. Complementation of the *rpfA-scr3097* deletion mutant. Schematic of the different complementation constructs. Hatched gray blocks indicate the location of the *rpfA* 5′ and 3′ UTRs, and black elbow arrows denote the transcriptional start sites of *rpfA* and *scr3097* as predicted using 5′ rapid amplification of cDNA ends[10] and RNA-seq,[14] respectively. Thick black lines above genes specify the location of the PCR products used to confirm the strain genotype. Primer pairs used are indicated above lines. Schematics were drawn to scale. The genotype of each complemented strain (Ex) was confirmed by PCR-amplifying the re-introduced gene from crude cell lysates. The complementation plasmid (+) and water (-) were used as positive and negative controls, respectively. *rpfA* alone, E120/pMC241; *scr3097* alone, E120/pMC242; *rpfA* + *scr3097*, E120/pMC243.

Supplementary References

1. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. Practical *Streptomyces* genetics. Norwich (England): The John Innes Foundation; 2000.
2. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A. 2000; 97:6640-6645.
3. MacNeil DJ, Gewain KM, Ruby CL, Dezeny G, Gibbons PH, MacNeil T. Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene. 1992; 111:61-68.
4. Redenbach M, Kieser HM, Denapaite D, Eichner A, Cullum J, Kinashi H, Hopwood DA. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. Mol Microbiol. 1996; 21:77-96.
5. Craney A, Hohenauer T, Xu Y, Navani NK, Li Y, Nodwell J. A synthetic *luxCDABE* gene cluster optimized for expression in high-GC bacteria. Nucleic Acids Res. 2007; 35:e46.
6. Sexton DL, St-Onge RJ, Haiser HJ, Yousef MR, Brady L, Gao C, Leonard J, Elliot MA. Resuscitation-promoting factors are cell wall-lytic enzymes with important roles in the germination and growth of *Streptomyces coelicolor*. J Bacteriol. 2015; 197:848-860.
7. Gust B, Challis GL, Fowler K, Kieser T, Chater KF. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci U S A. 2003; 100:1541-1546.
8. St-Onge RJ, Haiser HJ, Yousef MR, Sherwood E, Tschowri N, Al-Bassam M, Elliot MA. Nucleotide second messenger-mediated regulation of a muralytic enzyme in *Streptomyces*. Mol Microbiol. 2015; 96:779-795.
9. Paget MSB, Chamberlin L, Atrih A, Foster SJ, Buttner MJ. Evidence that the extracytoplasmic function sigma factor σ^E is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). J Bacteriol. 1999; 181:204-211.
10. Haiser HJ, Yousef MR, Elliot MA. Cell wall hydrolases affect germination, vegetative growth, and sporulation in *Streptomyces coelicolor*. J Bacteriol. 2009; 191:6501-6512.
11. Hindra, Moody MJ, Jones SE, Elliot MA. Complex intra-operonic dynamics mediated by a small RNA in *Streptomyces coelicolor*. PLoS One. 2014; 9:e85856.
12. Swiercz JP, Hindra, Bobek J, Haiser HJ, Di Berardo C, Tjaden B, Elliot MA. Small non-coding RNAs in *Streptomyces coelicolor*. Nucleic Acids Res. 2008; 36:7240-7251.
13. Darty K, Denise A, Ponty Y. VARNA: interactive drawing and editing of the RNA secondary structure. Bioinformatics. 2009; 25:1974-1975.
14. Moody MJ, Young RA, Jones SE, Elliot MA. Comparative analysis of non-coding RNAs in the antibiotic-producing *Streptomyces* bacteria. BMC Genomics. 2013; 14:558.