Supplementary Information

Cognate sigma and anti-sigma factors, and their relation to factors of the stressosome pathway in *Mycobacterium marinum*

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Supplementary methods

Analysis of the protein – protein interactions in the BACTH system

The BACTH vectors carrying the putatively interacting proteins were pairwise co-transformed into the *Escherichia coli* BACTH-strain BTH101 (F^-, *cya-99*, *araD139*, *galE15*, *galK16*, *rpsL1* [Str^R], *hsdR2*, *mcrA1*, *mcrB1*) and selected on LA-plates supplemented with streptomycin (100 µg/ml final concentration), ampicillin (100 µg/ml) and kanamycin (50 µg/ml) at 37ºC. Four colonies from each combination were streaked for single colonies on fresh plates of the same type. These were then used as the source for patching and plating for single colonies on LA media supplemented with the antibiotics as above and with the addition of 40 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Single colonies were patched on the same type of plates and incubated at 30ºC for up to three days. Colonies were also selected and grown for single colony formation under the same conditions. Patches and colonies were scored based on the development of blue color into three categories: negative (no blue color detected); weak/doubtful (color detected, but not strong or uniform across the patch or colonies); and positive (uniform blue color of intermediate to strong intensity of patches and colonies). The combinations of interacting proteins selected for single colony streaks were based on the patched plate results where at least three of the four replicates had to have positive or weak/doubtful scores of which at least two were positive. For the single colony streaks, a point scoring system was applied where strongly positive interactions were assigned three points, intermediately strong positive interactions two points, weakly positive interactions one point, very weak/doubtful interactions half a point, and negative interactions zero points. If the average across the four replicates were 2.5 or higher, the interactions were considered positive on the single colony level. See also Fig S2.

Co-immunoprecipitation, pulldown and Co-purification of interacting proteins

The anti-σ factors RsbW, RscA, anti-σ^{2997}, anti-σ^{3276}, and the predicted cytoplasmic domain of RscA (referred to as $RscA_{cyto}$) were cloned, with added 6xHis-tags, into the expression vector pET-28a(+). The corresponding σ factor were cloned in the same plasmid and fused with the FLAG-tag peptide in such a way that the σ and anti-σ factor pairs (or anti-σ and anti-anti-σ factor in the case of RsbW and RsbV) were co-expressed from the single T7-promoter of the pET-28a(+) plasmid. The plasmids were transformed into *E. coli* BL21 (DE3) cells. Cultures were initially inoculated with a single colony and grown in 20 ml Terrific Broth (TB) (with 50 µg/ml Kanamycin) at 37ºC overnight, diluted 100-fold into 500 ml fresh "TB Kanamycin" media, and grown at 37ºC until OD_{600} = 0.6 – 1.65 at which time IPTG was added to a final concentration of 1 mM to induce expression (by inducing T7 RNA polymerase). The cultures

were grown for an additional 24 hours at room temperature, harvested by centrifugation, and stored in 15 ml aliquots at -20°C. The cells were re-suspended in 1 ml of TBS (Tris buffered saline: 25 mM Tris, 137 mM NaCl, 5.0 mM KCl, 0.69 mM $CaCl_2$, 0.36 mM $MgCl_2$, 0.62 mM NaH_2PO_4, pH 7.4) for each 5 ml of original culture volume and cell lysates were prepared according to the Pierce™ Pull-Down PolyHis Protein:Protein Interaction Kit (Thermo Scientific) following the steps for bait protein preparation, bait protein immobilization, and bait-prey elution (since the prey protein was already expressed in the same cells as the bait protein, the separate steps for prey protein preparation and prey protein capture were excluded). Lysates were mixed with and equal volume of 2x Laemmli buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) and 12 µl aliquots were loaded on an Any-kD ready-made SDS-PAGE gel (Bio-Rad) together with a PAGE-ruler size marker (Thermo-Fisher) and run (at 300V constant voltage) until the 10 kD marker band reached the bottom of the gel. The proteins were visualized by incubating the gels in Oriole fluorescent gel stain (Bio-Rad) according to recommendations and imaged using a ChemiDoc MP imager (Bio-Rad).

To investigate the interaction between $RscA_{cyto}$ and σ^{C-1}, we constructed the plasmid pET-28a(+)-$RscA_{cyto}$-6×His- σ^{C-1}-FLAG, which encodes a C-terminal 6×His-tagged $RscA_{cyto}$ and a C-terminal FLAG-tagged σ^{C-1} was constructed. To generate the $RscA_{cyto}/\sigma^{C-1}$ construct, the $RscA_{cyto}/\sigma^{C}$ in pET-28a(+) was used as a template for restriction free cloning[84]. Briefly, the σ^{C-1} gene was amplified with primers with added overhangs complementary to the flanking regions of the σ^{C} gene in pET-28a(+). The resulting PCR-product was purified and used as a "megaprimer" to replace the σ^{C} gene in the construct generating a co-transcription construct with 6xHis-tagged $RscA_{cyto}$ and FLAG-tagged σ^{C-1}. To rule out potential artifacts introduced by epitope tags, we also constructed control plasmids, including plasmids lacking the FLAG tag, σ^{C-1}, or both. All these plasmids were transformed into *E. coli* BL21(DE3) cells. For protein expression, 1 ml of overnight culture was incubated into 50 ml of TB medium with 50 µg/ml kanamycin. Cultures were incubated at 37°C with shaking until an optical density at 600 nm (OD_{600}) of 0.6–0.8 was reached. The incubation temperature was then reduced to room temperature, and protein expression was induced with 1 mM IPTG. Cultures were grown overnight (~20 h). Cells from 15 ml of culture were harvested and lysed. A fraction of the cleared lysate was reserved as input. The remaining lysate was subjected to co-immunoprecipitation using the Pierce™ Pull-Down PolyHis Protein:Protein Interaction Kit (Thermo Scientific), following the manufacturer's instructions.

To study the interaction between $RskA_{cyto}$ and RsbUVW, we constructed the plasmid pETDuet-1-RsbUVW-6×His-$RskA_{cyto}$-FLAG encoding an N-terminal 6xHis-tagged RsbUVW and a C-terminal FLAG- $RskA_{cyto}$. The pETDuet-1 plasmid (Novagen) has a common promoter and two multiple cloning sites (MCS), each preceded by a T7 promoter, where the first MCS

enables an N-terminal fusion with a 6xHis-tag. The RsbUWV was cloned into the first MCS. In the other MCS the $RskA_{cyto}$ gene with a C-terminal FLAG-tag was cloned, simultaneously deleting the S-tag from pETDuet-1. This allows simultaneous expression of genes cloned in the two sites. For validation, we also generated two separate plasmids: pET-24a (+)-RsbUVW-6×His and pET-30a (+)-$RskA_{cyto}$-FLAG, expressing protein individually. Due to the poor expression of RsbUVW-6×His in BL21(DE3) cells (data not shown), all constructs were expressed in *E. coli* BL21 (DE3) pLysS cells to reduce basal expression and improve protein yield. Expression and harvest conditions were the same as described above. Co-immunoprecipitation was performed using the Pierce kit with minor modifications. Given the acidic nature of both proteins (predicted pI values: RsbUVW, 4.99; $RskA_{cyto}$, 4.12), the pH of the TBS buffer was adjusted from pH 7.4 to pH 6.0 to enhance protein stability. Additionally, the TBS buffer was supplemented with 25 mM glucose (to reduce protease release), 10% glycerol (to stabilize proteins), and 1 mM dithiothreitol (DTT) to maintain cysteine residues in a reduced state.

The constructs were transformed into *E. coli* BL21 (DE3) or *E. coli* BL21 (DE3) pLysS cells and grown, induced and harvested as above. Aliquots of 15 ml were lysed using the lysis buffer from the Pierce™ Pull-Down PolyHis Protein:Protein Interaction Kit (Thermo Scientific) following the steps for bait protein preparation. To 500 µl of crude lysate, 10 µl of mouse anti-6xHis antibody (Invitrogen, MA1-21315) or 10 µl of rabbit anti-FLAG antibody (Invitrogen, PA1-984B) was added and the samples were incubated overnight at 4°C with gentle agitation. Protein-G agarose beads (50 µl settled bead volume) were rinsed with 1 ml of Pierce Pull-Down PolyHis Protein:Protein Interaction Kit wash buffer and the lysate/antibody mixtures were transferred to the beads. The samples were incubated at 4°C for 30 min followed by 1.5 hr incubation at room temperature with constant gentle agitation. The protein-G/antibody/protein complexes were washed 5x for 2 min with wash buffer and 2 times with 1 x TE buffer (50 mM Tris, pH 7.9, and 1 mM EDTA) with 2 min pelleting of the complexes at 2000 x g between each wash. Finally, the proteins were eluted with 150 µl of elution buffer (50 mM Tris-HCl, pH 7.9; 150 mM NaCl; 0.1% NP40) at 65°C for 15 min. After saving the eluate, the beads were incubated for 5 min at 65°C with 1% SDS in 1 x TE buffer. The eluate from both procedures were combined and stored on ice.

Western blotting

The protein lysates were treated as above and duplicate blots (A and B) were prepared by running the RsbV/RsbW, $RscA_{cyto}/\sigma^C$, σ^{2997}/anti-σ^{2997}, and σ^{3276}/anti-σ^{3276} samples in parallel on duplicate Any-kD ready-made SDS-PAGE gels (Bio Rad) as above. The proteins were blotted to an Amersham Hybond low fluorescence PVDF blotting membrane (0.2 µm pore

size) following the manufacturer's instructions. The membranes were blocked using 0.1% (v/v) Tween 20 and 5% (w/v) dried skimmed milk in TBS (see above) for 1 hr. Primary antibodies targeting the FLAG-tag (PA1-984B; 1:500 dilution; from rabbit; blot A) or the 6xHis-tag (MA1-21315; 1:2000; from mouse; blot B) (Thermo-Fisher) were added to the membranes in the blocking solution and incubation continued for an additional hour. The blots were washed briefly in TBS containing 0.1% (v/v) Tween 20 (TBS-T) by incubation for 3x5 min. The blots were incubated with fluorescence conjugated secondary antibodies targeting the primary anti-FLAG peptide (A32734, Alexa Fluor PLUS 680 goat anti-rabbit IgG; 1:10000 dilution; from goat; blot A) or the anti-6xHis (A32723, Alexa Fluor PLUS 488 goat anti-mouse IgG; 1:20000 dilution; from goat; blot B) antibodies (Thermo-Fisher) in the blocking solution for 1 hr. The blots were washed in TBS-T as above. All incubations were carried out at room temperature. Fluorescence was detected using a ChemiDoc MP imager (Bio-Rad).

To validate and investigate the σ^{C-1}/$RscA_{cyto}$ and RsbUVW/$RskA_{cyto}$ interactions the protein samples were mixed with 2x SDS Laemmli sample buffer (see above), separated on 10% or 15% SDS-PAGE gel, depending on protein size. Proteins were transferred onto nitrocellulose membranes (Bio-Rad), and blocked with 5% non-fat dry milk in TBS containing 0.1% Tween-20 (TBST) for 1 hr at room temperature. The blocked membranes were incubated overnight at 4°C with primary antibodies diluted in blocking buffer anti-FLAG (Sigma, Cat No. F7425) and anti-His (Sigma, Cat No. V8012). Proteins were detected with the IRDye 680RD Goat anti-Rabbit IgG and IRDye 800CW Donkey anti-Mouse IgG secondary antibodies (Li-COR Biosciences). The Western blots were visualized using an Odyssey CLX imaging system (Li-COR Biosciences).

Dot blotting

The protein lysates were dot blotted onto an Amersham Hybond low fluorescence PVDF blotting membrane (0.2 µm pore size) using a Bio-Dot device (Bio-Rad). Blocking, washing and antibody incubation was performed as described above except that primary antibody incubation was done overnight and 5 instead of 3 washes were carried out after each incubation. In addition, in a second experiment, both primary antibodies were added to the same blot, as was the two secondary antibodies in the final detection step.

Gene expression analysis

M. marinum (*Mmar*) CCUG 20998 cells were grown and exposed to various stresses as previously described[11]. RNA from duplicate samples was extracted and further processed to remove traces of DNA as previously described[11] and submitted for whole transcriptome sequencing at the SNP&SEQ Technology Platform (HiSeq2000—Illumina—platform, Uppsala

University) and at GATC Biotech (Konstanz, Germany; Illumina technology), or converted to cDNA for analysis by quantitative Real-Time PCR (qRT-PCR) as described in[11,26]. The data was processed as previously described[11].

RNA sequencing reads were mapped with the genome *Mmar* CCUG 20998 as reference[34] and transcript abundance was calculated as described below. We used the average mRNA levels for the replicates in each stress condition and calculated percentage distribution of the σ and anti-σ factor mRNAs. Deviations were calculated from the replicates and are represented as "Standard Error". We filtered out genes that were not expressed in any samples before differential gene expression analysis. Differential gene expression analysis was performed using DESeq2[83] considering the stress-less control (unstressed condition) as a control with a corrected *P-value* < 0.05 FDR, false discovery rate[11].

Analysis of the σ and anti-σ factor mRNA levels as a function of time after heat, cold, and microaerobic stress induction was performed using qRT-PCR on an ABI 7300 system using default settings and probe-based detection as described in[11] using the stress-less control as the calibrator sample and the standard curve method of quantification. The small subunit ribosomal RNA (16S rRNA) was used as internal standard. Primers and probes are listed in Supplementary Table S1 and in[11]. The values were then averaged and normalized to the exponential phase sample (time 0 h, immediately before stress induction). The errors for the values at each time-point were calculated by combining the errors of the individual series (six combined measurements of two biological replicates) using standard methods[11]. The 0 h time point is common to all series and consists of five biological replicates (15 combined measurements). We used a two-tailed Student's t-test to calculate significant changes at the different time points compared to the pre-induction 0 h time point. Fold-change values were $\log_2$-transformed before testing for significance to fit the values into a normal distribution. The corresponding p-values are listed in Table S4. We also tested for any correlation between the σ and anti-σ factor mRNA curves by calculating their Pearson correlation coefficients (listed in Table S4) using the Excel Analysis toolpack.

References numbering refers to the numbering shown in the list of references in the main text.

Supplementary Tables

Table S1 Primers and probes for qRT-PCR

Primer/probe	Sequence (5' –> 3')	Comment
FP15123-rscA-qRT-fw2	TTTTGAGCGTCTATGTGGTTGTG	Mm rscA primer
FP15124-rscA-qRT-rv2	GGTCAGGACCCGCGAAGT	Mm rscA primer
Mm-rscA probe2	6FAM-ATGCGCTGTCCGGTG-MGBNFQ	Mm rscA probe
FP15125-rsdA-qRT-fw1	GCCGACAGTACCTGCTAAACG	Mm rsdA primer
FP15126-rsdA-qRT-rv1	CGGATCGCGCTTTTCG	Mm rsdA primer
Mm-rsdA probe1	6FAM-TGAATCAGCTCAACACC-MGBNFQ	Mm rsdA probe
FP1221-	TCGGCGCGCGTTCT	Mm rseA primer
FP1222-	GGCGCATCGCTTTGAGA	Mm rseA primer
Mm_rseA-probe	6FAM-TGGCTTCCTGCTCAGTT-MGBNFQ	Mm rseA probe
FP1301-asigHfw-qRT	CCGGATGCCTGAAGCATTAC	Mm rshA primer
FP1302-asigHrv-qRT	CCCTGACACTTGGTGGCAAT	Mm rshA primer
MM-asigHpb	6FAM-CTCGAGGAGCGGATC-MGBNFQ	Mm rshA probe
FP1303-asigKfw-qRT	GGGTGTGTTGGTGATGAACAAT	Mm rskA primer
FP1304-asigKrv-qRT	CGACCAGCCACATCTGATAGAC	Mm rskA primer
MM-asigKpb	6FAM-CCGTCTCCGGGCAC-MGBNFQ	Mm rskA probe
FP1219-	GCCGCGGTGTTTTTGG	Mm rslA primer
FP1220-	AACCGGCGCAGAAGCA	Mm rslA primer
Mm_rslA-probe	6FAM-TGCTGGTTGGTGTGGTAG-MGBNFQ	Mm rslA probe
FP15121-rsmA-qRT-fw1	CACCGCGATGCACATCA	Mm rsmA primer
FP15122-rsmA-qRT-rv1	CGAGGACCTGGTCGTGTGA	Mm rsmA primer
Mm-rsmA probe1	6FAM-CCTCCAGTGATTCCG-MGBNFQ	Mm rsmA probe
FP1307-asig3276fw-qRT	GGTGACCGACTATCTCGAGGAT	Mm a3276 primer
FP1308-asig3276rv-qRT	CCTCTGGTGTGCTGTTCGAA	Mm a3276 primer
MM-asig3276pb	6FAM-CTCGACGAGTCTGAC-MGBNFQ	Mm a3276 probe
FP1329-Ec-16Sfw-qRT	CCAGGGCTACACACGTGCTA	Ec 16S rRNA primer
FP1330-Ec-16Srv-qRT	TCTCGCGAGGTCGCTTCT	Ec 16S rRNA primer
EC-16Spb	6FAM-AATGGCGCATACAAA-MGBNFQ	Ec 16S rRNA probe
MSMEG-qRT sigA Fw	GCCCGCACCATCCGTAT	Ms sigA primer
MSMEG-qRT sigA Rv	ATACGGCCGAGCTTGTTGAT	Ms sigA primer
ULP051110 sigA	6FAM-CCGGTGCACATGGT-MGBNFQ	Ms/Mm sigA probe
MSMEG-qRT sigB Fw	GCGTTGACACCGATCTGGAT	Ms sigB primer
MSMEG-qRT sigB Rv	GTCTGCGGCATTGAGCAAT	Ms sigB primer
Mm_sigB-probe	6FAM-TGCGCGTGTATCTG-MGBNFQ	Ms/Mm sigB probe
MSMEG-qRT rshA Fw	CGGGTGAAACGGCTGATC	Ms rshA primer
MSMEG-qRT rshA Rv	AGCCGCTCACGCAAACTATC	Ms rshA primer
MSMEG rshA Pb	6FAM-CCGCCAAGTGCAGC-MGBNFQ	Ms rshA probe

Mm: *M. marinum*. Ms: *M. smegmatis*. Ec: *E. coli*.

Table S2 Presence of protein domains found in known anti-σ factors

Topology	1-84;143-148;196-237 inside	1-84 inside				1-90 inside	1-112 inside	1-106 inside		
Length (a.a.)	237	314	134	172	101	232	248	249	88	94
	rscA	***rsdA***	***rseA***	***rsbW***	***rshA***	***rskA***	***rslA***	***rsmA***	**a2997**[p]	**a3276**
***M. marinum* CCUG 20998**[q]	zf-HC2 [6-36][a]; 4xTM [85-105;125-142;149-166;178-195][b]; *YlaC [1-237][c], e-value 4.98e-81; antisig_ RsrA [2-36][d]; RsiW [3-138][e]	*RsdA_SigD_bd [14-58][f], e-value 2.80e-15; 1xTM [85-107][b]	(*RsiW [34-85][e], e-value 0.02); (zf-HC2 [42-71][a]); (antisig_ RsrA [41-87][d])	*RsbW [24-170][h], e-value 2.73e-20; PTHR 35526 [29-167][g]	*anti_SigH_actin [16-98], e-value 4.85e-32; zf-HC2 [26-56][a]; antisig_ RsrA [22-98][d]; RsiW [34-60][e]; YlaC [34-56][c]	*RskA [67-227][k], e-value 2.53e-25; PRK 13920 [16-226][i]; 1xTM [91-113][b]	*zf-HC2 [27-52][a] e-value 8.92e-07 ; RsiW [27-235][e]; 1xTM [113-135][b]	*TH1 [146-192][m], e-value 0.01; (RsiW [10-121]) 1xTM [107-129][b]	*(YlaC [10-59][c], e-value 0.05); (zf_HC2 [35-45][a]); (antisig_ RsrA [33-46][d])	*zf-HC2 [19-50][a], e-value 1.83e-05; antisig_ RsrA [16-49][d]; YlaC [14-83]; RsiW [16-82][e]
***M. tuberculosis* H37Rv**	zf-HC2 [52-83][a]; 4xTM [130-150;170-187;194-211;223-240][b]; *YlaC [46-282][c]; RsiW [49-207][e] antisig_ RsrA [48-82][d]	RsdA_SigD_bd [13-58][f]; 1xTM [86-107][b]	(zf-HC2 [45-74][a]); (antisig_ *RsrA [44-90][d])	*RsbW [94-240][h]; PTHR 35526 [99-242][g]	zf-HC2 [26-56][a]; antisig_ RsrA [22-81][d]	*RskA [67-227][k]; PRK 13920 [16-222][i]; 1xTM [91-111][b]	zf-HC2 [33-58][a]; antisig_ RsrA [24-71][d]; *RsiW [1-239][e]; 1xTM [115-136][b]	Nd[n]	–	–
Length (a.a.)	283	299	154	242	101	233	251	255		
Topology	1-129;188-193;241-282 inside					1-90 inside	1-114 inside			

Combined results of the NCBI CDD search and the InterProScan search. Domains in parentheses are below the default threshold e-value of the CDD search (0.01). Values within brackets [ ] indicate where the domains are located within the protein. The domain hit e-values are indicated for the best hits (indicated with *). Protein length and membrane topology are also indicated. **a.** A putative zinc-finger motif (Pfam entry PF13490). **b.** Transmembrane domain. **c.** Domain similar to inner membrane protein YlaC in *E.coli* (PF10777). **d.** Anti-σ^R factor, mycothiol system, RsrA (IPR024020). **e.** Transmembrane transcriptional regulator (anti-σ factor RsiW) (COG5662). **f.** Anti-σ^D factor RsdA to σ factor binding region (PF16751). **g.** Unnamed family containing anti-σ factor RsbW. **h.** Serine-protein kinase RsbW (IPR010193). **i.** Putative anti-σ^E protein; Provisional (PRK13920). **k.** Anti-σ^K factor RskA (PF10099). **m.** TH1 is a highly conserved but uncharacterized metazoan protein (PF04858). **n.** No domains detected. **p.** From *M. marinum* NCTC2275 (absent from strain CCUG 20998). **q.** *M. marinum* M and NCTC2275 have almost identical amino acid sequences as CCUG 20998 for these proteins and were not tested separately. − marks protein not present.

Table S3 Distribution of a2997 and a3276 in mycobacteria

	Identity	Coverage	E-value
a2997			
M. marinum Europe, E11	100%	100%	1E-58
M. marinum M	99%	100%	4E-58
M. marinum MB2	97%	100%	2E-56
Mycobacterium liflandii 128FXT	97%	100%	7E-57
Mycobacterium sp. 012931	97%	100%	7E-57
Mycobacterium pseudoshottsii L15	97%	100%	7E-57
Mycobacterium ulcerans subsp *shinshuense*	97%	100%	7E-57
Mycobacterium ulcerans Agy99, Harveyi	95%	100%	1E-56
Mycobacterium gordonae	79%	98%	5E-45
Mycobacterium kansasii ATCC 12478	76%	100%	1E-44
Mycobacterium gastri 'Wayne'	76%	100%	1E-44
Mycobacterium europaeum	75%	100%	6E-44
Mycobacterium sp	76%	100%	2E-43
Mycobacterium sp	78%	98%	3E-43
Mycobacterium interjectum	74%	100%	4E-43
Mycobacterium sp	74%	100%	4E-43
Mycobacterium colombiense	76%	100%	4E-43
Mycobacterium tuberculosis TKK-01-0051	74%	100%	2E-42
Mycobacterium triplex	74%	98%	6E-42
Mycobacterium scrofulaceum	73%	100%	7E-42
Mycobacterium intracellulare	72%	100%	1E-41
Mycobacterium indicus pranii	70%	100%	5E-41
Mycobacterium yongonense	70%	100%	5E-41
Mycobacterium avium subsp. *avium* 2285 (S)	70%	100%	5E-41
Mycobacterium sp	70%	100%	5E-41
Mycobacterium chimaera	70%	100%	1E-40
Mycobacterium malmoense	68%	100%	5E-40
Mycobacterium simiae	77%	92%	9E-40
Mycobacterium sherrisii	78%	90%	3E-39
Mycobacterium szulgai	71%	95%	3E-39
Mycobacterium bohemicum	71%	98%	4E-39
Mycobacterium intermedium	67%	100%	6E-39
Mycobacterium asiaticum	69%	97%	3E-37
Mycobacterium avium (all subsp.)	67%	100%	9E-37
Mycobacterium genavense	75%	100%	8E-35
Mycobacterium abscessus	69%	94%	2E-34
Mycobacterium kyorinense	64%	92%	6E-34
Mycobacterium celatum	63%	90%	3E-32

	Identity	Coverage	E-value
a3276			
M. marinum M, Europe, E11, MB2	Identical	100%	6E-61
M. marinum MB2	99%	100%	5E-60
Mycobacterium liflandii 128FXT	99%	100%	5E-60
Mycobacterium ulcerans subsp *shinshuense*	99%	100%	5E-60
Mycobacterium sp. 012931	99%	100%	5E-60
Mycobacterium pseudoshottsii L15	97%	79%	8E-45
Mycobacterium ulcerans Agy99, Harveyi	96%	79%	3E-44
Mycobacterium arupense	62%	95%	1E-35
Mycobacterium sp. (8 in total)	62%	95%	1E-35
Mycobacterium abscessus FLAC031	62%	95%	1E-35
Mycobacterium avium	67%	96%	2E-35
Mycobacterium kansasii ATCC 12478	65%	90%	8E-35
Mycobacterium chlorophenolicum	65%	90%	8E-35
Mycobacterium abscessus	65%	90%	8E-35
Mycobacterium canariasense	65%	90%	8E-35

Values from BLASTp searches *vs* the NCBI non-redundant protein database as per 29 Sept. 2016.

Table S4. Statistics for the σ factor/anti-σ factors mRNA level time curves.

P values for difference compared to the 0 timepoint.

	time	*rscA*	*rsdA*	*rseA*	*rsbW*	*rshA*	*rskA*	*rslA*	*rsmA*	*asig3276*
Heat	1 h	0.808	0.151	0.059	0.628	0.296	0.046	0.607	0.029	0.125
	4 h	0.811	0.147	0.008	0.245	0.918	0.025	0.440	0.023	0.047
	6 h	0.922	0.359	0.004	0.274	0.689	0.053	0.146	0.035	0.037
	12 h	0.545	0.662	0.018	0.296	0.402	0.200	0.135	0.051	0.059
	24 h	0.113	0.948	0.009	0.880	0.111	0.588	0.008	0.144	0.058
Cold	1 h	0.560	0.016	0.020	0.588	0.009	0.578	0.148	0.203	0.033
	4 h	0.593	0.050	0.013	0.974	0.004	0.198	0.075	0.452	0.040
	6 h	0.495	0.039	0.008	0.920	0.002	0.150	0.041	0.246	0.034
	12 h	0.528	0.049	0.009	0.928	0.002	0.055	0.035	0.218	0.047
	24 h	0.307	0.058	0.006	0.683	0.002	0.052	0.019	0.166	0.020
Micro-aerobic	9 h	0.062	0.003	0.004	0.011	0.001	0.009	0.075	0.001	0.009
	1 d	0.037	0.002	0.004	0.004	0.001	0.005	0.050	3.2E-04	0.005
	3 d	0.014	0.001	0.001	0.001	0.001	0.002	0.007	1.9E-04	0.001
	6 d	0.005	3.0E-04	0.001	4.7E-04	3.7E-04	0.001	0.002	3.1E-04	3.6E-04
	12 d	0.003	0.003	4.5E-04	2.8E-04	1.2E-04	0.001	0.001	7.4E-05	3.6E-04

$p < 0.05$	$p < 0.01$	$p < 0.001$

Correlation coefficients between σ factor/ anti-σ factors time curves

	time	*rscA/ sigC*	*rscA/ sigC_1*	*rsdA/ sigD*	*rseA/ sigE*	*rsbW/ sigF*	*rshA/ sigH*	*rskA/ sigK*	*rslA/ sigL*	*rsmA/ sigM*	*asig3276/ sig3276*
Heat	0 - 24 h	-0.012	0.056	0.831	0.975	0.936	0.999	0.935	0.976	-0.041	0.975
	1 - 24 h									0.969	
	4 - 24 h	0.999	0.969								
Cold	0 - 24 h	0.183	-0.846	0.935	0.714	0.435	0.853	0.896	0.728	-0.762	0.980
	1 - 24 h							0.964		-0.892	
Micro-aerobic	0 - 24 h	0.989	0.977	1.000	0.996	0.998	0.979	0.989	0.995	0.993	0.995

|Corr. Coeff.|	< 0.75	0.75 - 0.85	0.85 - 0.95	> 0.95

Figure S1 Secondary structure conservation of anti-σ factors containing a zf-HC2/ZAS motif.

The *M. marinum* (Mm) and *M. tuberculosis* (Mtb) anti-σ factors containing a predicted zf-HC2/ZAS zinc finger motif were compared with that of known zf-HC2/ZAS containing anti-σ factors ChrR from *Rhodococcus sphaeroides* (Rs) and RsrA from *Streptomyces coelicolor* (Sc), as well as the *M. tuberculosis* RslA for which the crystal structure has been solved[41]. The secondary structures were predicted using JPred 4[40]. Amino acids with orange and pink background shading were predicted to be in helical structures. The predicted transmembrane region is indicated with gray background shading, and the conserved residues in the Cys/His$X_{23\text{-}26}$HisX_3CysX_2Cys zf-HC2/ZAS motif are shown with a yellow background. The boxed residues correspond to α helices 1 to 4 in[39].

Fig S1

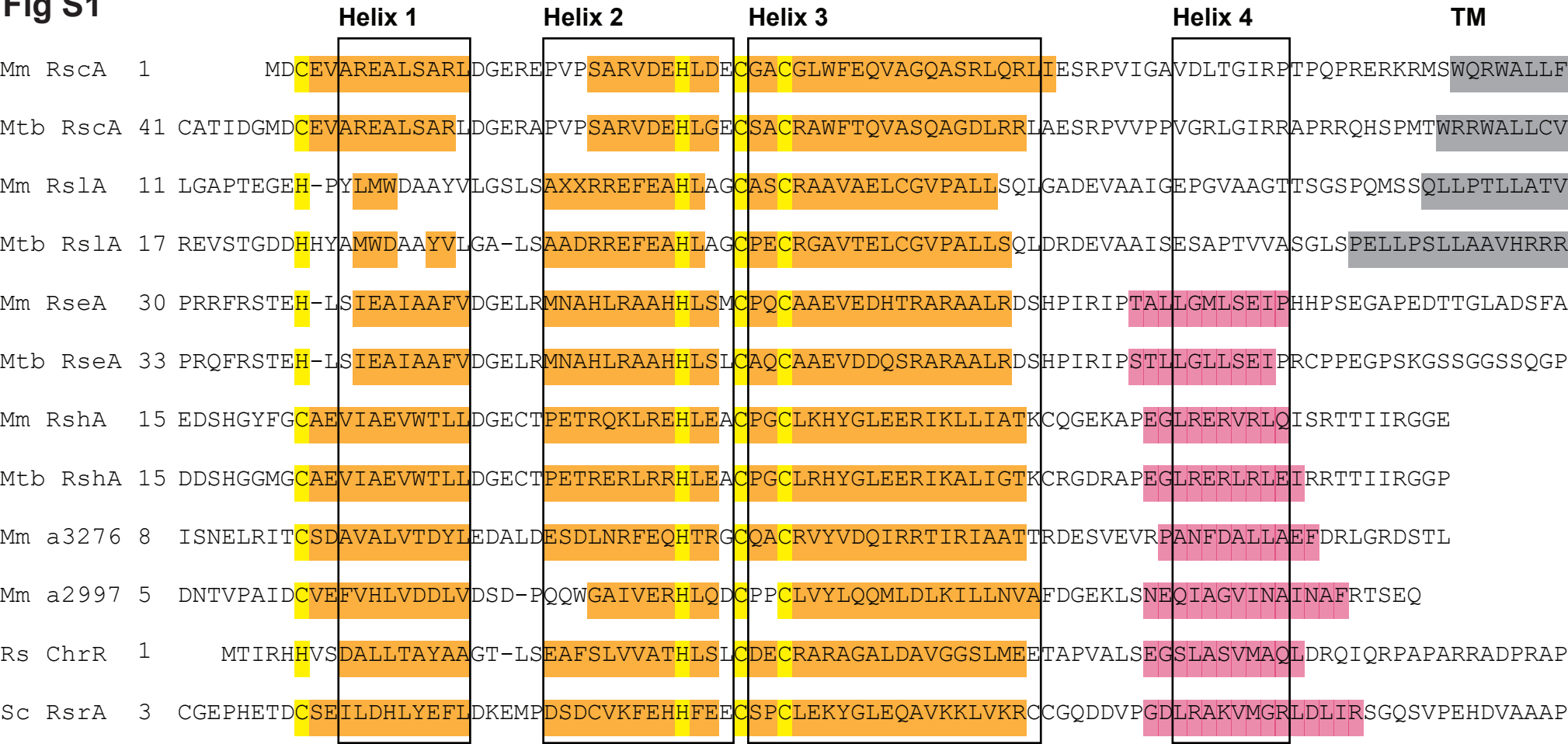

Helix 1
Helix 2
Helix 3
Helix 4
TM
Mm RscA 1 MDCEVAREALSARLDGEREPVPSARVDEHLDECGACGLWFEQVAGQASRLQRLIESRPVIGAVDLTGIRPTPQPRERKRMSWQRWALLF
Mtb RscA 41 CATIDGMDCEVAREALSARLDGERAPVPSARVDEHLGECSACRAWFTQVASQAGDLRRLAESRPVVPPVGRLGIRRAPRRQHSPMTWRRWALLCV
Mm RslA 11 LGAPTEGEH-PYLMWDAAYVLGSLSAXXRREFEAHLAGCASCRAAVAELCGVPALLSQLGADEVAAIGEPGVAAGTTSGSPQMSSQLLPTLLATV
Mtb RslA 17 REVSTGDDHHYAMWDAAYVLGA-LSAADRREFEAHLAGCPECRGAVTELCGVPALLSQLDRDEVAAISESAPTVVASGLSPELLPSLLAAVHRRR
Mm RseA 30 PRRFRSTEH-LSIEAIAAFVDGELRMNAHLRAAHHLSMCPQCAAEVEDHTRARAALRDSHPIRIPTALLGMLSEIPHHPSEGAPEDTTGLADSFA
Mtb RseA 33 PRQFRSTEH-LSIEAIAAFVDGELRMNAHLRAAHHLSLCAQCAAEVDDQSRARAALRDSHPIRIPSTLLGLLSEIPRCPPEGPSKGSSGGSSQGP
Mm RshA 15 EDSHGYFGCAEVIAEVWTLLDGECTPETRQKLREHLEACPGCLKHYGLEERIKLLIATKCQGEKAPEGLRERVRLQISRTTIIRGGE
Mtb RshA 15 DDSHGGMGCAEVIAEVWTLLDGECTPETRERLRRHLEACPGCLRHYGLEERIKALIGTKCRGDRAPEGLRERLRLEIRRTTIIRGGP
Mm a3276 8 ISNELRITCSDAVALVTDYLEDALDESDLNRFEQHTRGCQACRVYVDQIRRTIRIAATTRDESVEVRPANFDALLAEFDRLGRDSTL
Mm a2997 5 DNTVPAIDCVEFVHLVDDLVDSD-PQQWGAIVERHLQDCPPCLVYLQQMLDLKILLNVAFDGEKLSNEQIAGVINAINAFRTSEQ
Rs ChrR 1 MTIRHHVSDALLTAYAAGT-LSEAFSLVVATHLSLCDECRARAGALDAVGGSLMEETAPVALSEGSLASVMAQLDRQIQRPAPARRADPRAP
Sc RsrA 3 CGEPHETDCSEILDHLYEFLDKEMPDSDCVKFEHHFEECSPCLEKYGLEQAVKKLVKRCCGQDDVPGDLRAKVMGRLDLIRSGQSVPEHDVAAAP

Figure S2 BACTH schematic outline.

The bacterial adenylate cyclase based two-hybrid (BACTH) system[42] utilizes that the *Bordetella pertussis* adenylate cyclase can be separated into two independent domains, T18 (orange) and T25 (green), which work together to produce cyclic AMP (cAMP, purple) only when brought into proximity of each other. In **A**, both domains are covalently linked to each other like in the native enzyme and cAMP is produced. In **B** and **C**, both domains are brought into proximity of each other by two interacting proteins (dark blue and light blue, respectively) fused to the T18 and T25 domains, respectively, and cAMP is produced. In **C**, the light blue protein is membrane bound with the interacting domain on the cytoplasmic side of the membrane (inner membrane, IM, in dark gray; cell wall, CW, in light gray). In **D**, **E**, and **F**, no cAMP is produced. In **D**, the interacting domain of the light blue membrane bound protein, and thereby also the T25 domain, is facing the extracellular environment and is unable to interact with the intracellularly located dark blue protein (and the fused T18 domain) since they are physically separated. In **E**, both domains are expressed intracellularly, but since they have no affinity for each other and are not fused to any interacting proteins they do not produce cAMP. In **F**, the T18 and T25 domains are fused to proteins, which do not interact (yellow and light blue, respectively) resulting in no cAMP production (essentially the same situation as in **E**). Production of cAMP results in β-galactosidase production, which can be detected on an LA + X-Gal plate since cAMP forms a complex with the catabolite activator protein CAP (turquoise), and the cAMP-CAP complex binds to the promoter region (a purple arrow marks the promoter and an open box immediately to the left of the arrow corresponds to the cAMP-CAP binding site) and activates β-galactosidase expression. The large filled circle in the bottom middle represents an LA plate supplemented with X-Gal. In situations **A**, **B**, and **C**, cAMP and thereby also β-galactosidase are produced resulting in X-Gal being metabolized generating a blue indicator dye (sectors **A**, **B**, and **C**, respectively) indicating positive interactions. In situations **D**, **E**, and **F**, no cAMP (no β-galactosidase) is produced leaving X-Gal un-metabolized and the bacterial colonies colorless (sectors **D**, **E**, and **F**). Importantly, the *E. coli* BTH101 strain carries an inactivated *cya* (encoding adenylate cyclase) gene.

Fig S2

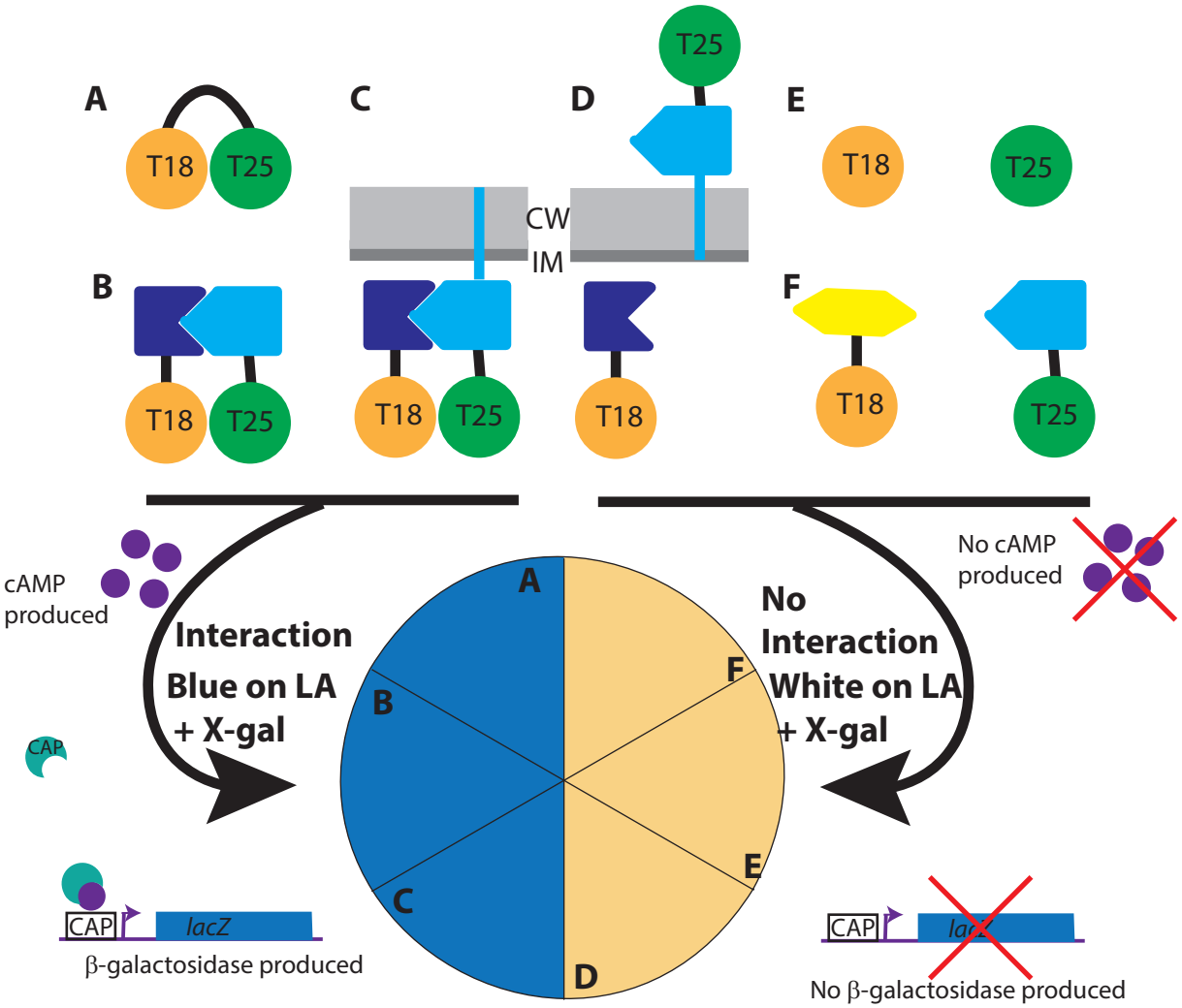

A
T18
T25
C
D
T25
E
T18
T25
CW
IM
B
T18
T25
T18
T25
T18
F
T18
T25
cAMP
produced
Interaction
Blue on LA
+ X-gal
No cAMP
produced
No
Interaction
White on LA
+ X-gal
CAP
A
B
C
D
E
F
CAP
lacZ
β-galactosidase produced
CAP
No β-galactosidase produced

Figure S3 σ and anti-σ factor interactions.

The σ and anti-σ factors were cloned in the BACTH system and co-expressed in *E. coli* BTH101 cells and patched on LA + X-gal plates containing the appropriate antibiotics. Blue colored patches indicate positive interactions. Patching was done for 4 biological replicates and a representative plate is shown. The underlined patches were selected for testing for single colony formation shown in Fig 3 and the positive and negative controls were the same as in Fig 3.

A For each σ/anti-σ factor pair, the vector combinations were, from left to right (σ/anti-σ): pKT25/pUT18; pKT25/pUT18C; pKNT25/pUT18; pKNT25/pUT18C; pUT18/pKT25; pUT18C/pKT25; pUT18/pKNT25; pUT18C/pKNT25.

Note, for SigC/RscA the results are presented in the two first rows as indicated where the first corresponds to the first four vector combinations while the second row corresponds to the last four vector combinations listed above. For SigM/RsmA, starting at the horizontal line, the first two patches represent the two first vector combinations, while the third row represents the last six vector combinations listed above.

B For SigC_1/RscA the vector combinations used were as indicated in the figure and grown on a separate plate compared to the rest of the σ factor/anti-σ factor combinations tested. For comparison, the plate also shows the previously detected[26] positive interactions between the RsbW (W) anti-σ factor and the RsbV (V) anti-anti-σ factor in the order indicated (with #) below the plate.

Fig S3

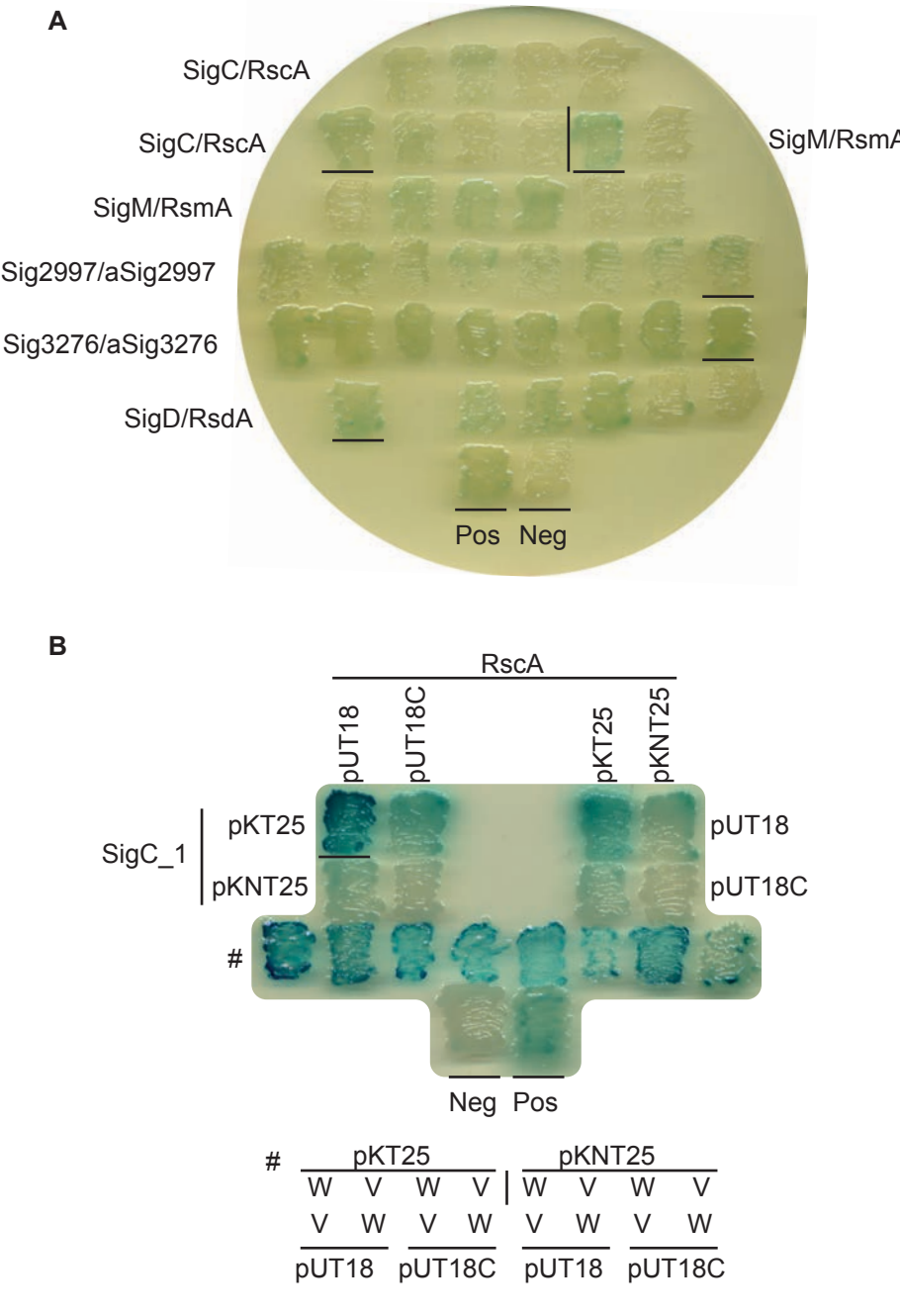

A
SigC/RscA
SigC/RscA
SigM/RsmA
SigM/RsmA
Sig2997/aSig2997
Sig3276/aSig3276
SigD/RsdA
Pos
Neg
B
RscA
pUT18
pUT18C
pKT25
pKNT25
SigC_1
pKT25
pKNT25
pUT18
pUT18C
#
Neg
Pos
#
pKT25
pKNT25
W V W V W V W V
V W V W V W V W
pUT18
pUT18C
pUT18
pUT18C

Figure S4 Complementary images to main figures 3B-D.

A Refers to Fig 3B and the image shows the full-size of the cropped image used in Fig 3B (Oriole Fluorescent stain). Exposure settings automatic for faint bands. The image was inverted from black/white to white/black in Fig 3. For details, see figure legend 3. Lanes 1-10 and M (marker) are marked for orientation.

B Refers to Fig 3B and the image shows the full-size of the cropped image used in Fig 3B (Oriole Fluorescent stain). Exposure settings, 0.1 s. The image was inverted from black/white to white/black in Fig 3. For details, see figure legend 3. Lanes 1-10 and M (marker) are marked for orientation.

C Refers to Fig 3C (left panel, lanes 1-4 and marker lane M). The image shows the full-size of the cropped image used in Fig 3C (anti-6xHis antibody). Exposure (low) settings automatic for faint bands. The image was inverted from black/white to white/black in Fig 3. For details, see figure legend 3.

D Refers to Fig 3C (left panel, lanes 1-4 and marker lane M). The image shows the full-size of the cropped image used in Fig 3C (anti-6xHis antibody). Exposure (increased) settings, 5 s. The image was inverted from black/white to white/black in Fig 3. For details, see figure legend 3.

E Refers to Fig 3C (right panel, lanes 7-10 and marker lane M). The image shows the full-size of the cropped image used in Fig 3C (anti-FLAG antibody). Exposure (low) settings automatic for faint bands. The image was inverted from black/white to white/black in Fig 3. For details, see figure legend 3.

F Refers to Fig 3C (right panel, lanes 7-10 and marker lane M). The image shows the full-size of the cropped image used in Fig 3C (anti-FLAG antibody). Exposure (increased) settings automatic for faint bands. The image was inverted from black/white to white/black in Fig 3. For details, see figure legend 3.

G Proteins tagged with 6xHis- or FLAG-tags were pulled down (along with interacting partners) using anti-6xHis or anti-FLAG antibodies and the samples were dot-blotted to a low fluorescence background PVDF blotting membrane. Replicate blots were probed for either the 6xHis-tag (top blot) or the FLAG-tag (bottom blot). The affinity tag used to make the pull-down is indicated at the top of each blot (either 6xHis or FLAG). The insets show the results of replicate blots since the larger blot was damaged. The spots are numbered 1 to 8 as indicated, see also Fig S4H-K.

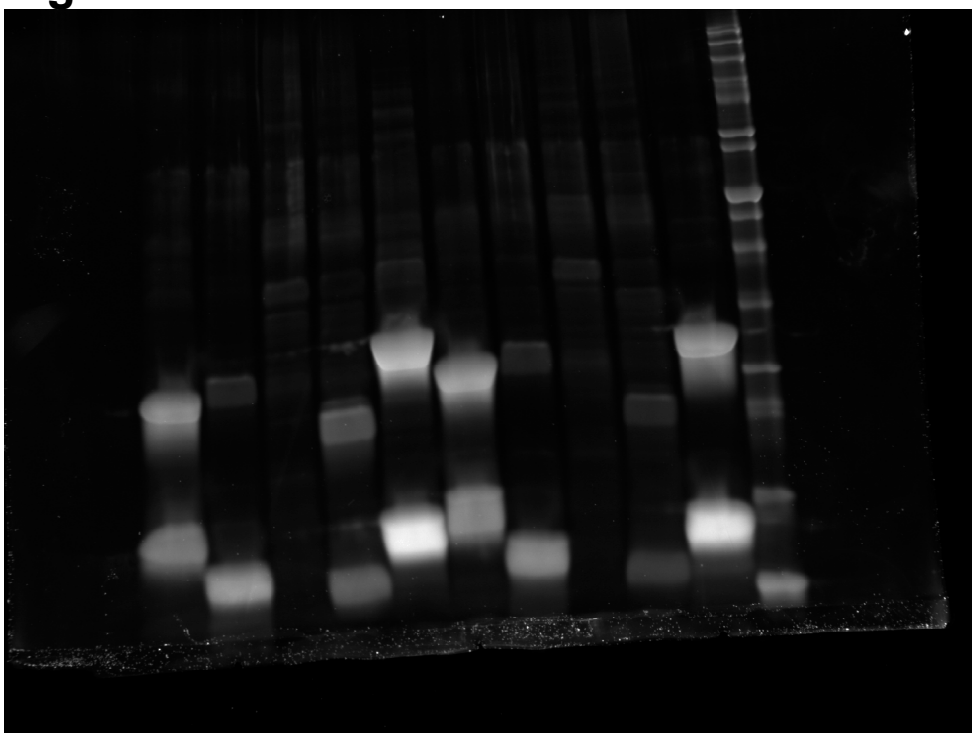
H Refers to Fig S4G (upper panel, spots 1 to 6). The image shows the full-size of the cropped image used in Fig S4G (anti-6xHis antibody). The image was inverted from black/white to white/black in Fig S4G. For details, see figure legend S4G.

I Refers to Fig S4G (upper panel, spots 7 and 8). The image shows the full-size of the cropped image used in Fig S4G (anti-6xHis antibody). The image was inverted from black/white to white/black in Fig S4G. For details, see figure legend S4G.

J Refers to Fig S4G (lower panel, spots 1 to 6). The image shows the full-size of the cropped image used in Fig S4G (anti-FLAG antibody). The image was inverted from black/white to white/black in Fig S4G. For details, see figure legend S4G.

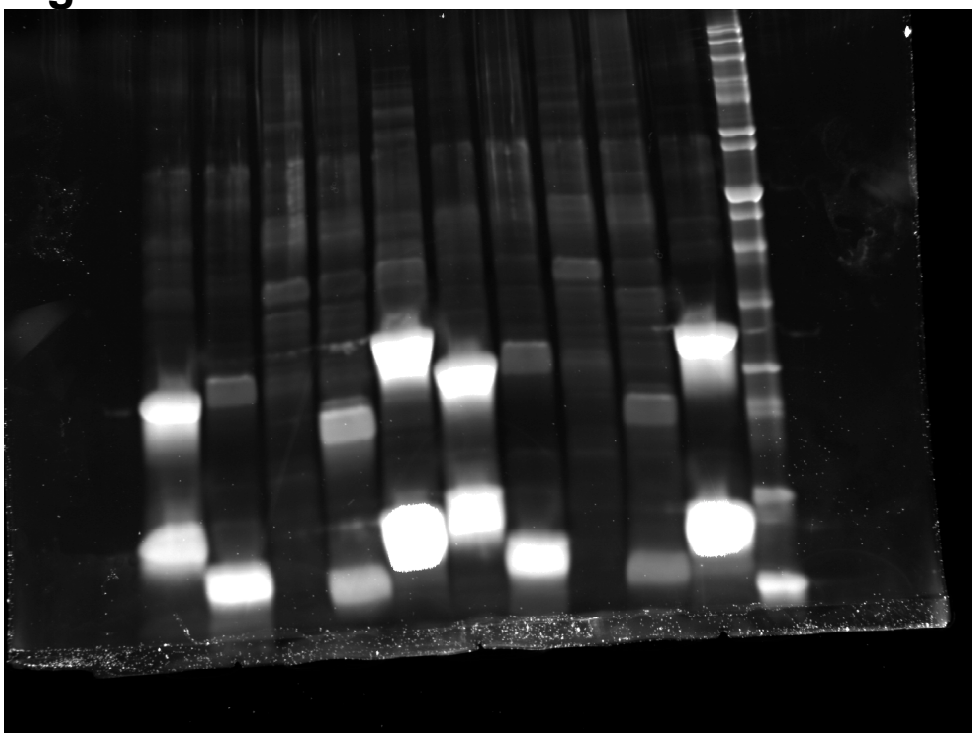
K Refers to Fig S4G (lower panel, spots 7 to 8). The image shows the full-size of the cropped image used in Fig S4G (anti-FLAG antibody). The image was inverted from black/white to white/black in Fig S4G. For details, see figure legend S4G.

Fig S4A



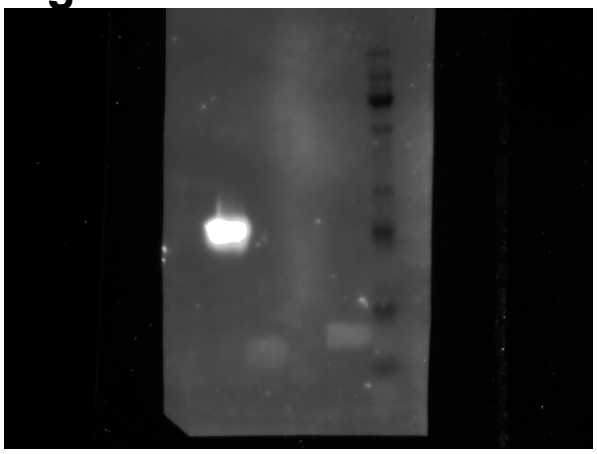
Lane 1 2 3 4 5 6 7 8 9 10 M

Fig S4B



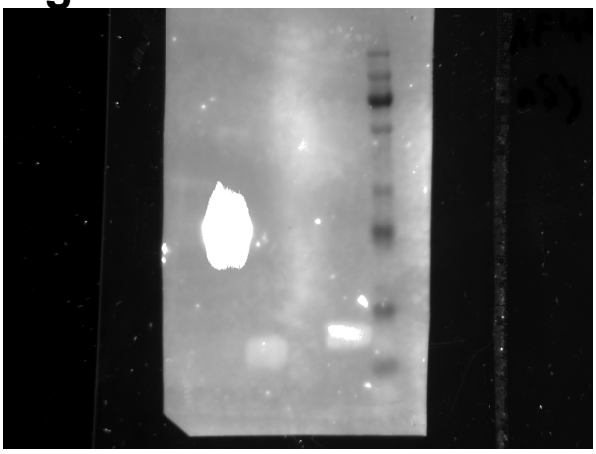
Lane 1 2 3 4 5 6 7 8 9 10 M

Fig S4C



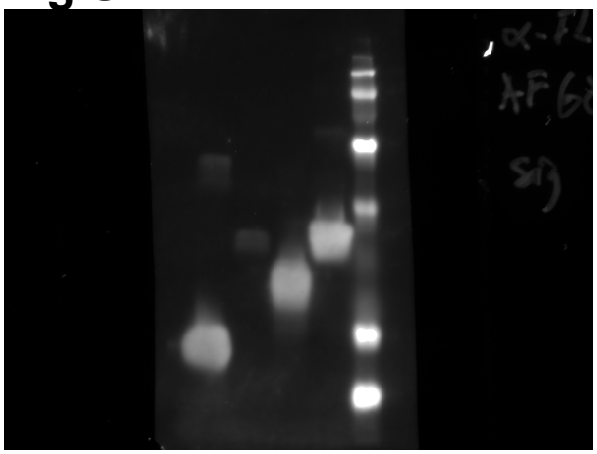
Lane 1 2 3 4 M

Fig S4D



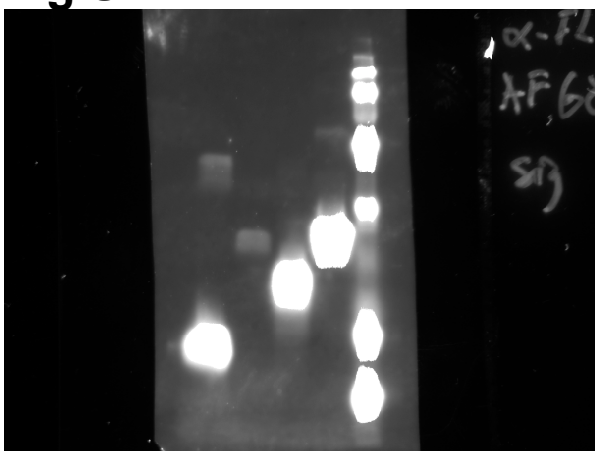
Lane 1 2 3 4 M

Fig S4E



Lane 7 8 9 10M

Fig S4F



Lane 7 8 9 10M

Fig S4G

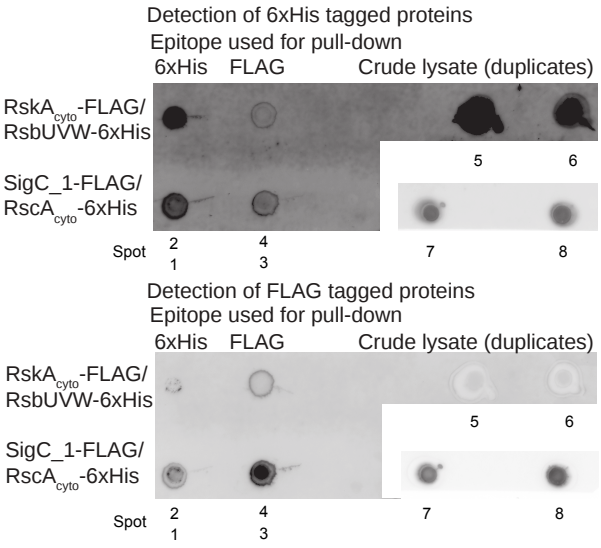


Fig S4H

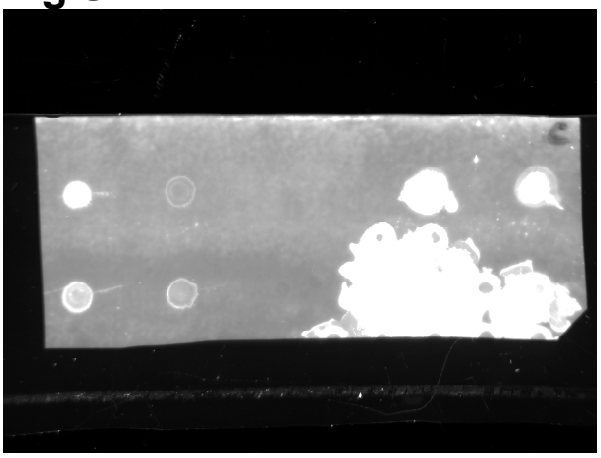


Fig S4J

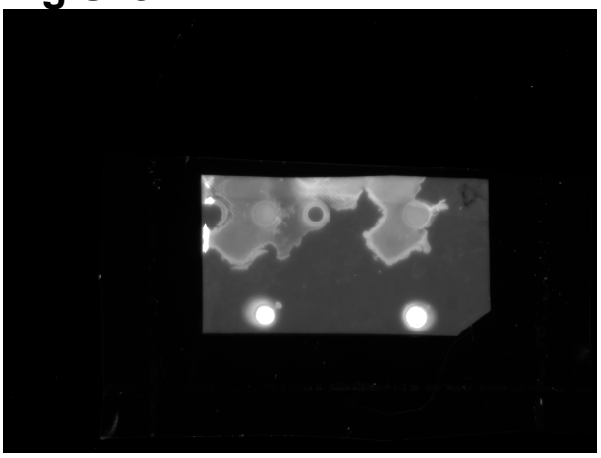


Fig S4I

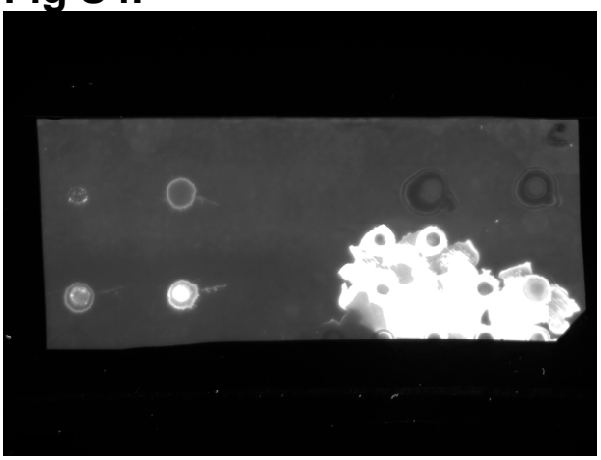


Fig S4K

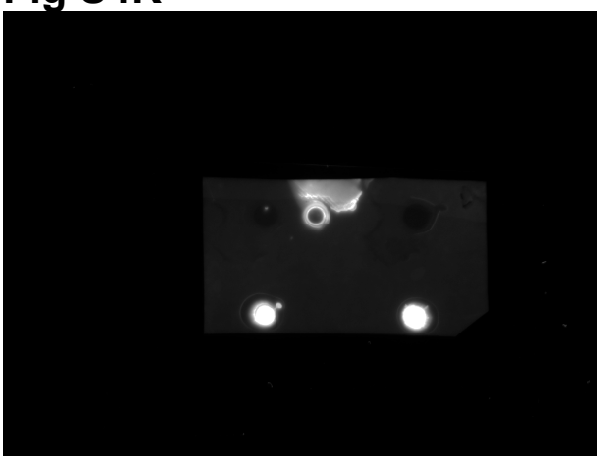
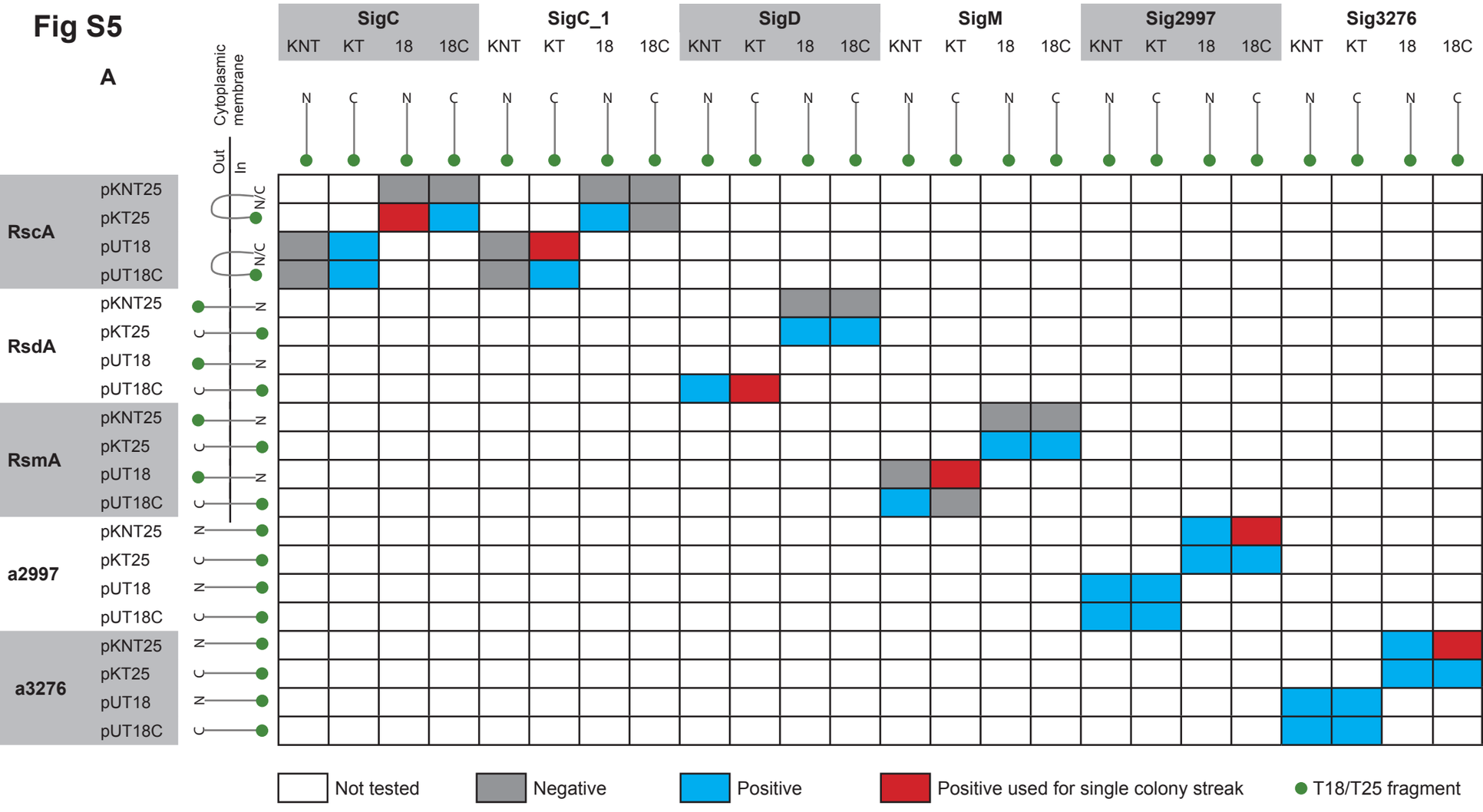


Figure S5 σ and anti-σ factor interactions.

A The indicated σ and anti-σ factors were cloned in the BACTH system and co-expressed in *E. coli* BTH101 cells and patched on LA + X-gal plates containing the appropriate antibiotics. Blue colored boxes indicate that the patches had a blue color, which was interpreted as a positive interaction. Patching was done for four biological replicates (of which one representative replicate is shown in Fig S3; see also main Fig 3A) and a representative summary is shown. The red boxes (also positive) were selected for testing for the single colony streaks shown in Fig 3 (main text). The cartoons indicate a simplified structure and membrane topology of the fusion proteins where N and C are the free N- and C-termini of the σ and anti-σ factors (gray lines), and the green dots are the adenylate cyclase T25 and T18 domains, which need to interact to activate β-galactosidase expression (see Fig S2). The black line represents the cytoplasmic membrane and the in- and outside where "in" corresponds to the cytoplasmic side. The vector constructs pKNT25 and pUT18 have the anti-σ C-termini fused to T25 and T18 while pKT25 and pUT18C have the anti-σ N-termini fused to T25 and T18 as indicated. **B** The cartoon summarizes the positive interactions between the putatively membrane bound anti-σ factors and the intracellular σ factors shown in **A** in relation to the predicted membrane topology.

Fig S5


A
SigC
SigC_1
SigD
SigM
Sig2997
Sig3276
KNT KT 18 18C
Cytoplasmic membrane
Out
In
RscA
RsdA
RsmA
a2997
a3276
pKNT25
pKT25
pUT18
pUT18C
N/C
Not tested
Negative
Positive
Positive used for single colony streak
T18/T25 fragment

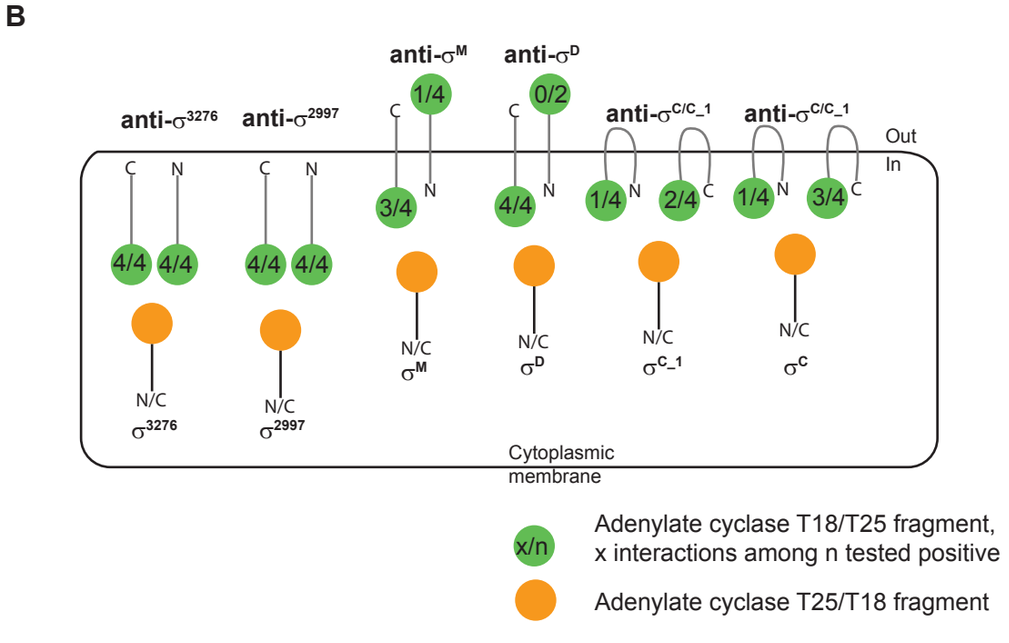

B
anti-σ3276
anti-σ2997
anti-σM
anti-σD
anti-σC/C_1
anti-σC/C_1
Out
In
1/4
0/2
3/4
4/4
1/4
2/4
1/4
3/4
N/C σ3276
N/C σ2997
N/C σM
N/C σD
N/C σC_1
N/C σC
Cytoplasmic membrane
x/n
Adenylate cyclase T18/T25 fragment, x interactions among n tested positive
Adenylate cyclase T25/T18 fragment

Figure S6 Ratio of absolute levels of σ factor to anti-σ factor pair mRNAs after exposure to various stress conditions.

Mmar^{CCUG} mRNA read counts from RNA-Seq data after exposure to various stress conditions. Each box plot represents the RNA-Seq mRNA transcript (raw) read counts ratio of the σ factor gene to their cognate anti-σ factor gene plotted on $\log_2$ scale (y-axis). On the y-axis, positive means higher mRNA levels for the σ factor mRNA whereas negative values correspond to the higher levels of the anti-σ factor mRNA. On the x-axis, each colored box corresponds to different stress conditions as indicated.

Fig S6

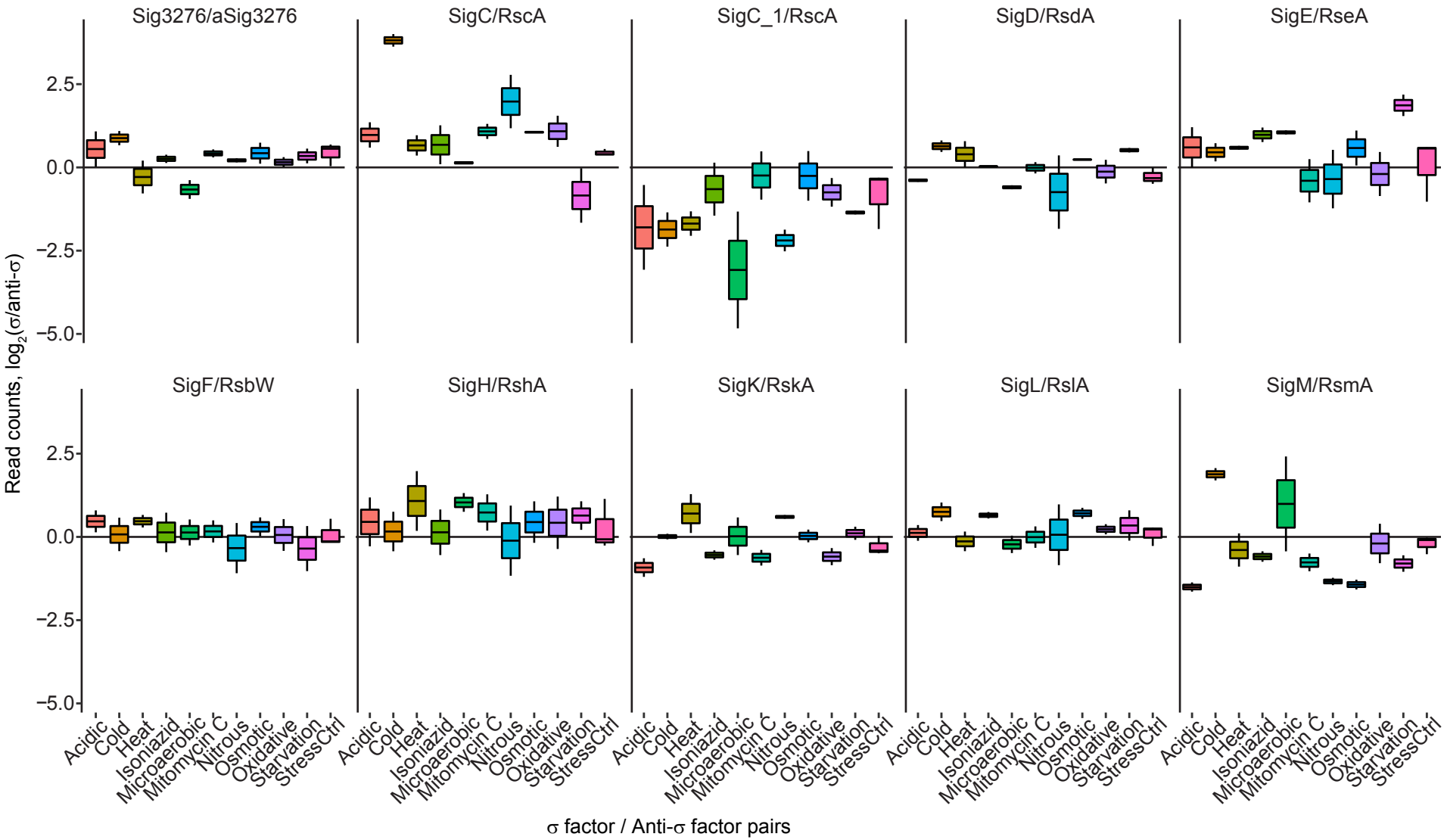
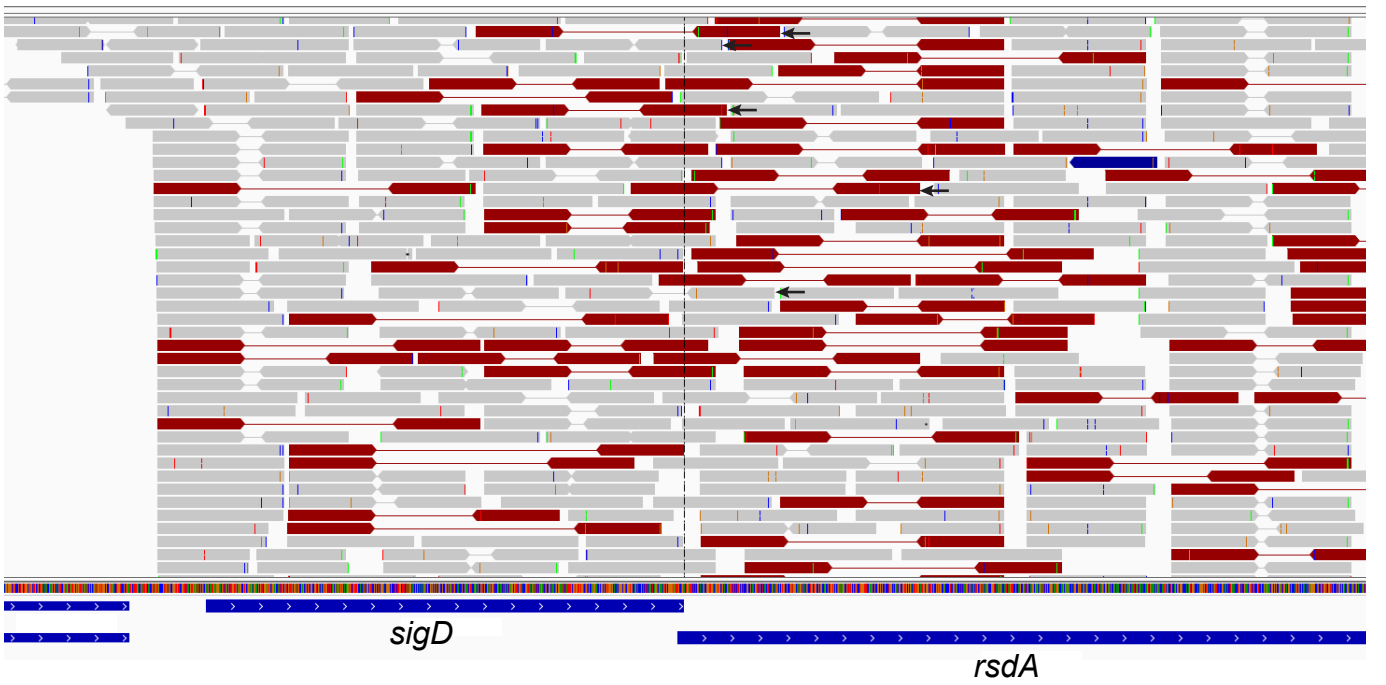

Sig3276/aSig3276
SigC/RscA
SigC_1/RscA
SigD/RsdA
SigE/RseA
SigF/RsbW
SigH/RshA
SigK/RskA
SigL/RslA
SigM/RsmA
2.5
0.0
−2.5
−5.0
Read counts, log2(σ/anti-σ)
Acidic
Cold
Heat
Isoniazid
Microaerobic
Mitomycin C
Nitrous
Osmotic
Oxidative
Starvation
StressCtrl
σ factor / Anti-σ factor pairs

Figure S7 Co-transcription of σ/anti-σ factor pairs.

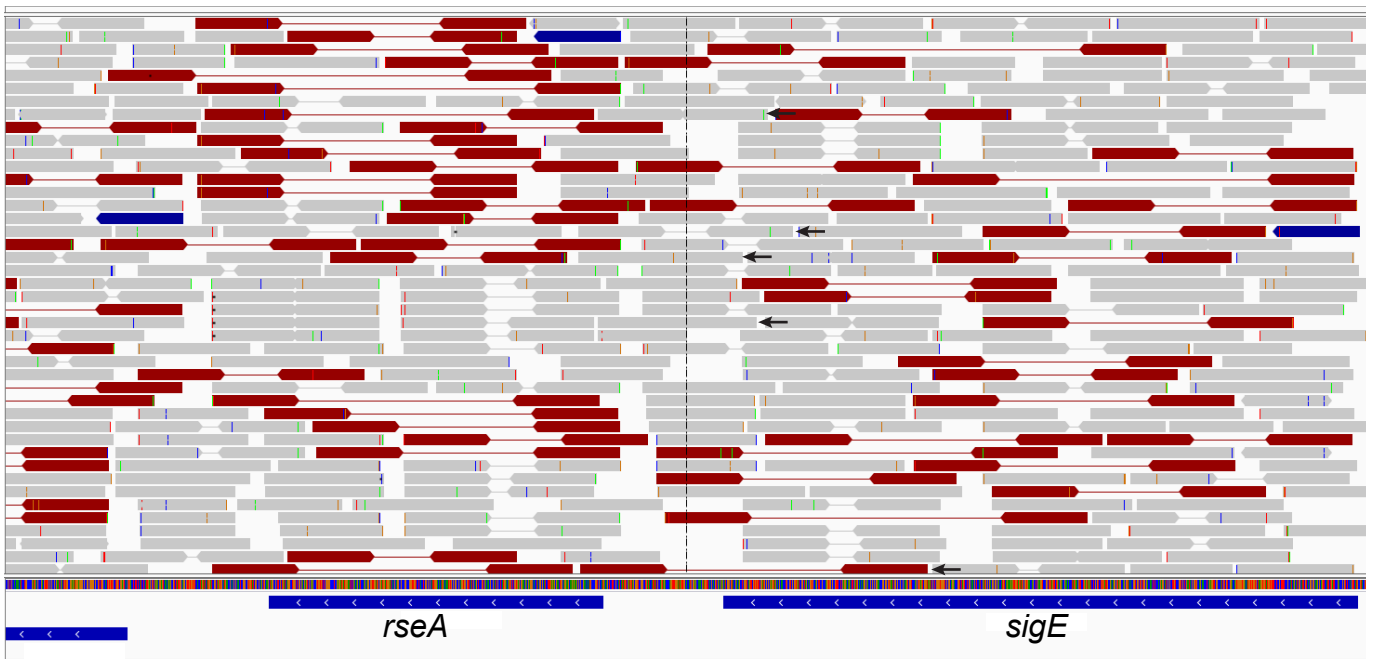
A snapshot of the aligned paired end reads (indicated in grey and red) for the (**A)** σ^D/*rsdA*, (**B)** σ^E/*rseA*, (**C)** σ^F/*rsbW*, (**D)** σ^H/*rshA* coding regions. Reads and paired end reads that start in one of the genes and end in the other while spanning the region between them are indicated with arrows. The vertical line indicates the junction between the genes. Paired end reads colored in red are separated by at least 300 base pairs. The bottom track indicates the respective genes (blue).

Fig S7

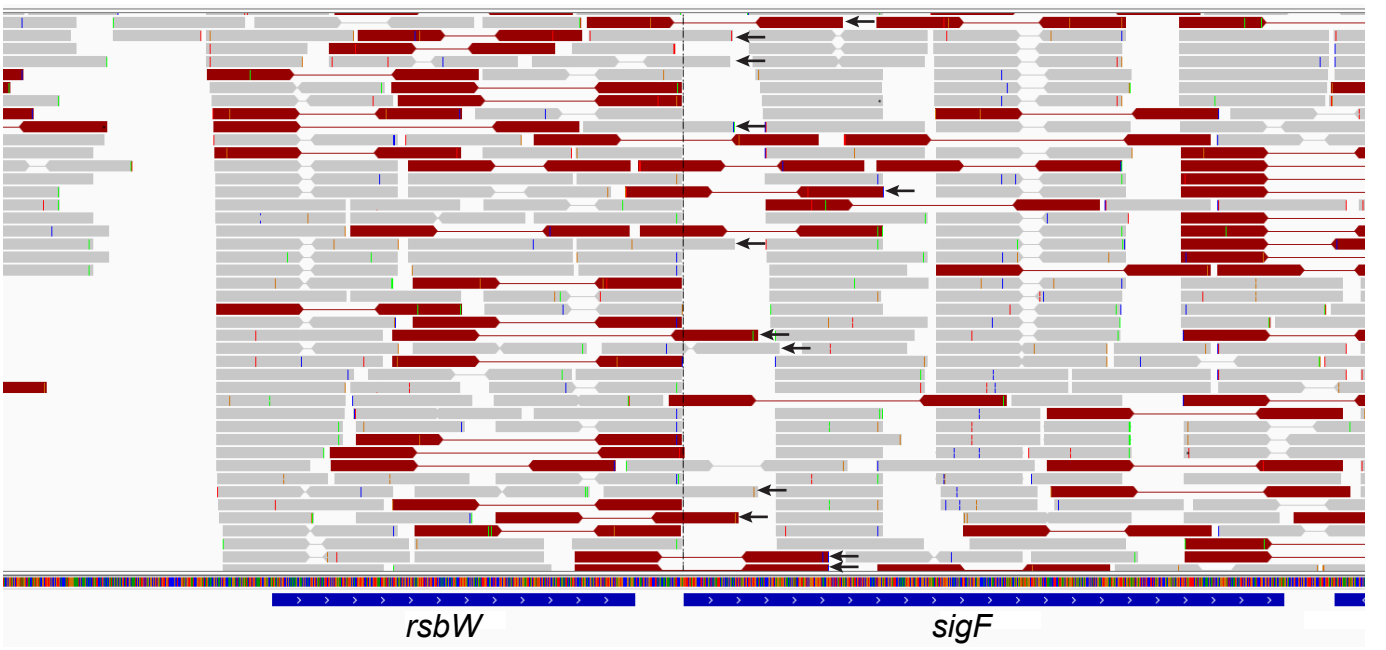
A



B



C



D

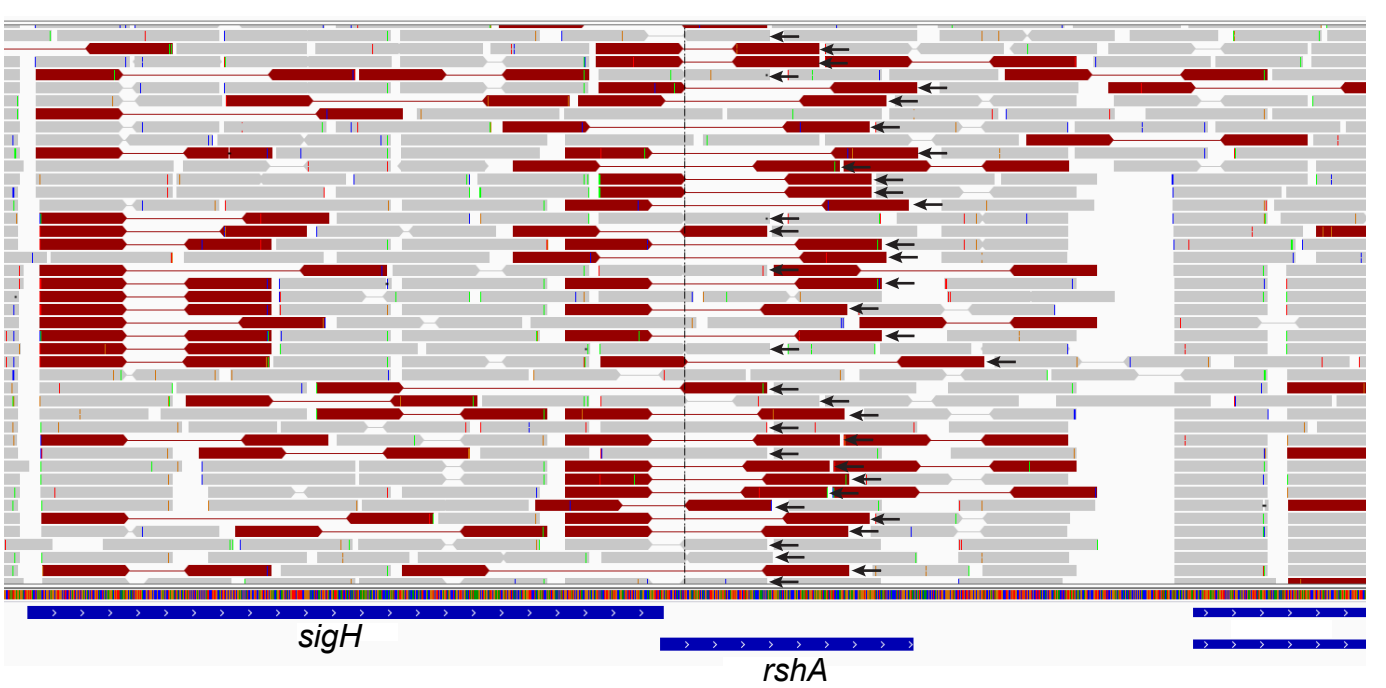


Figure S8 Co-transcription of σ/anti-σ factor pairs.

A snapshot of the aligned paired end reads (indicated in grey and red) for the (**A**) σ^{3276}/anti-σ^{3276}, (**B**) σ^{K}/*rskA*, (**C**) σ^{L}/*rslA*, and (**D**) σ^{M}/*rsmA* coding regions. Reads and paired end reads that start in one of the genes and end in the other while spanning the region between them are indicated with arrows. The vertical line indicates the junction between the genes. Paired end reads colored in red are separated by at least 300 base pairs. The bottom track indicates the respective genes (blue).

Fig S8

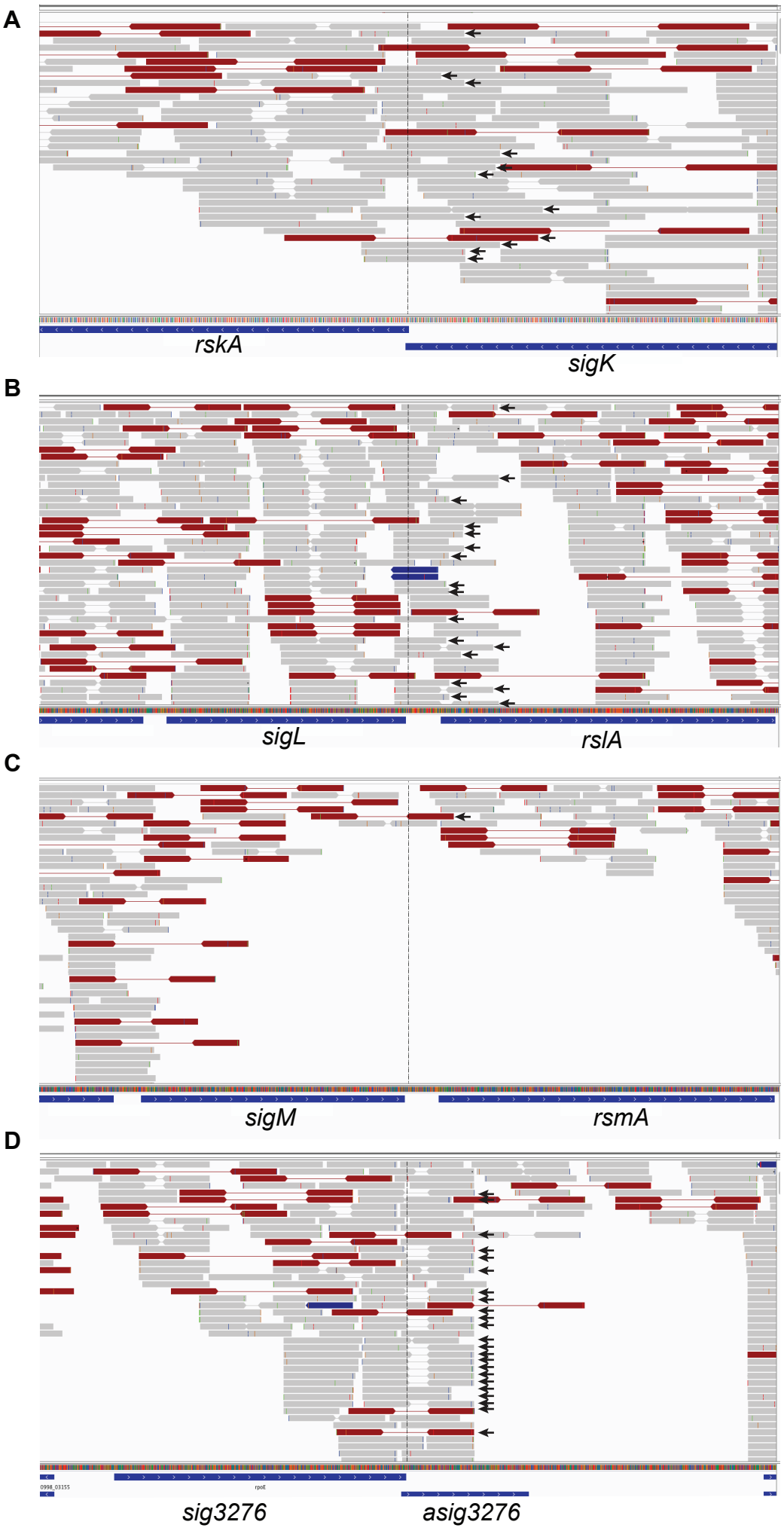


Figure S9 mRNA levels for σ/anti-σ factor pairs upon exposure to heat, cold and microaerobic stress as function of time

The mRNA levels for the σ/anti-σ factor pairs as a function of time after stress induction for heat (hours), cold (hours), and microaerobic (days) stresses. The values are given as average of two induced biological replicates compared to five un-induced biological replicates with standard errors as indicated (note, for the majority of the time points the errors were too low to be seen). The *sigC*_1 is indicated with dotted lines in the *sigC*_1 *sigC rscA* panel. The data relating to σ factor mRNA levels taken from[11].

Fig S9

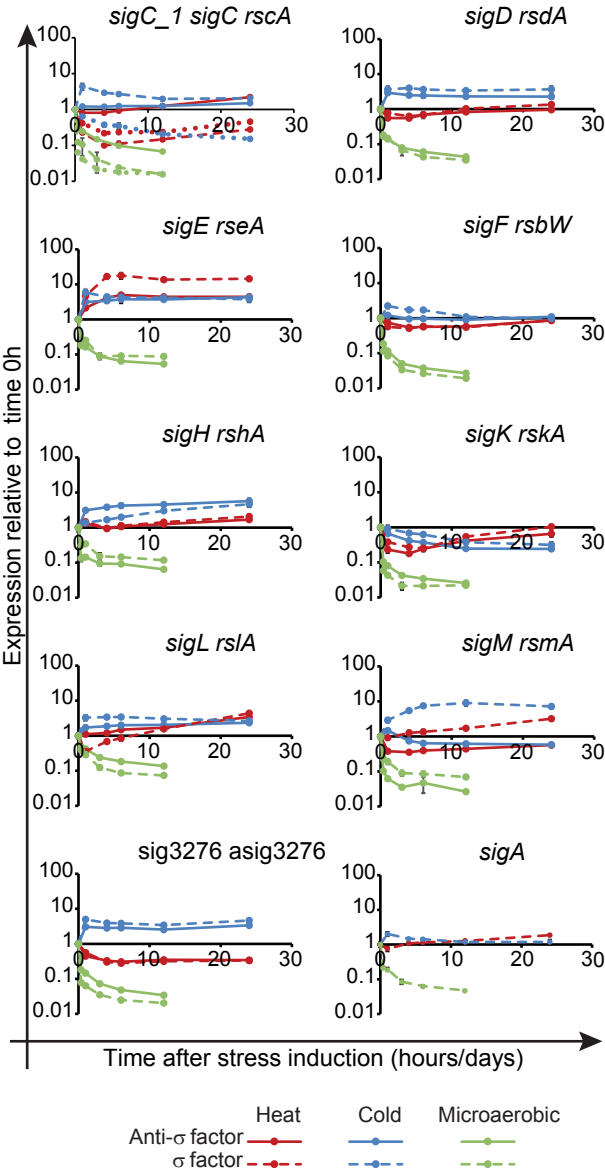
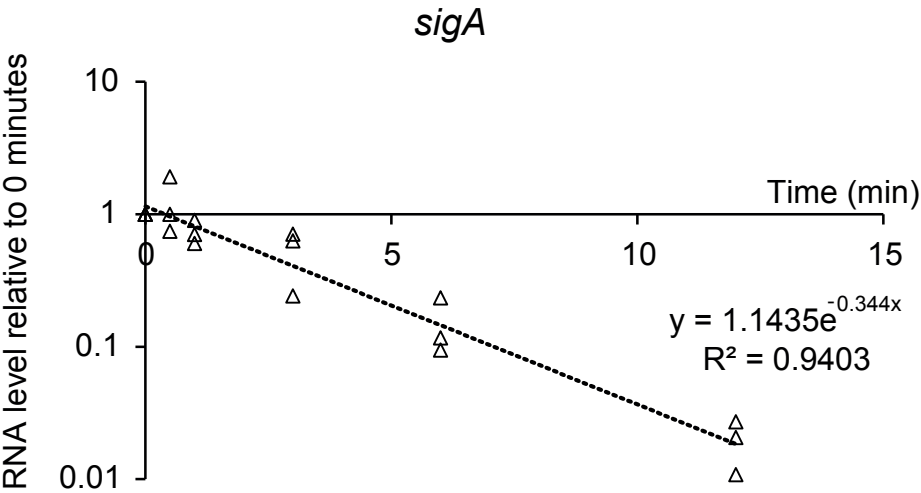

sigC_1 sigC rscA
sigD rsdA
sigE rseA
sigF rsbW
sigH rshA
sigK rskA
sigL rslA
sigM rsmA
sig3276 asig3276
sigA
100
10
1
0.1
0.01
0
10
20
30
Expression relative to time 0h
Time after stress induction (hours/days)
Heat
Cold
Microaerobic
Anti-σ factor
σ factor

Figure S10 Determination of *sigA* and *sigB* mRNA half-lives.

The half-lives of *sigA* and *sigB* mRNAs were determined by quantitative real-time PCR from cells spiked with equal amounts of exponentially growing *E. coli* MG1655 cells. The x-axis refers to the time after rifampicin addition (at 0 min). The results of three independent biological replicates are shown and the graph shows an exponential fit to the data with the R^2 value as indicated and the equation of the fitted graph from which the half-lives were calculated. This equation (from *sigA*) was also used to calibrate the *sigA* mRNA levels from the mRNA sequencing data (see Methods for details).

Fig S10


sigA
RNA level relative to 0 minutes
10
1
0.1
0.01
Time (min)
0
5
10
15
y = 1.1435e-0.344x
R² = 0.9403

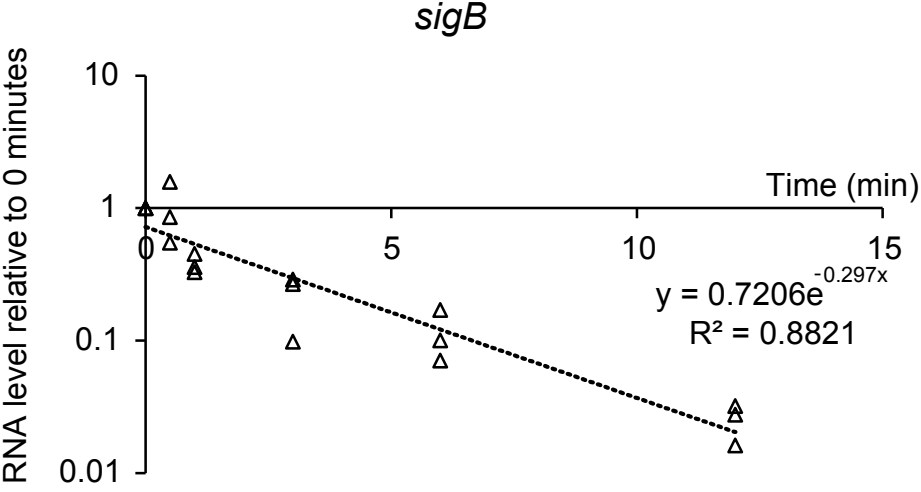
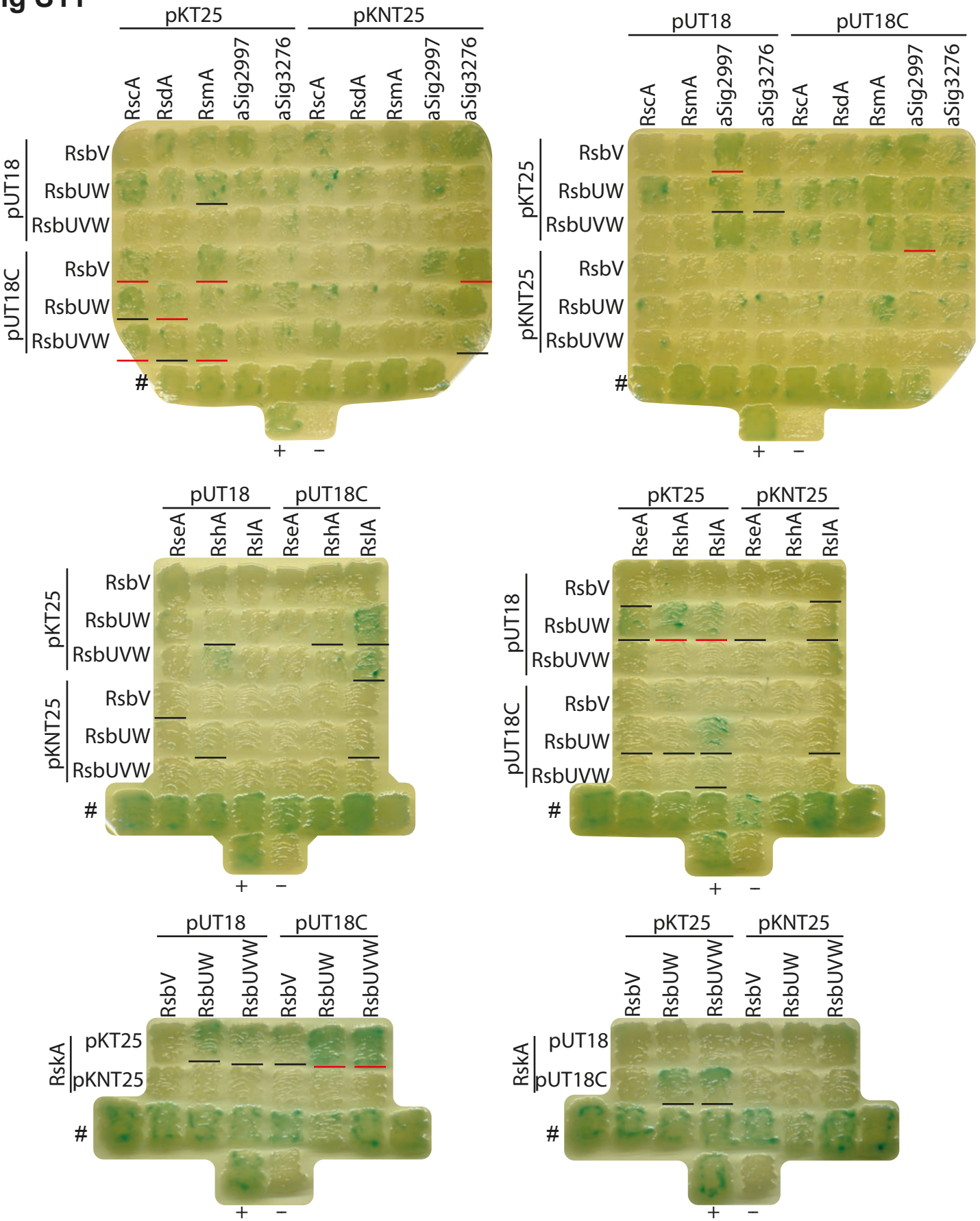

sigB
RNA level relative to 0 minutes
10
1
0.1
0.01
Time (min)
0
5
10
15
y = 0.7206e-0.297x
R² = 0.8821

Figure S11 Anti-σ factors interacting with stressosomal proteins.

The anti-σ factors cloned in the BACTH system were co-expressed with RsbV, RsbUW, and RsbUVW (previously cloned in the BACTH system[26]) in *E. coli* BTH101 cells and patched on LA + X-gal plates containing the appropriate antibiotics. Blue colored patches indicate positive interactions. Patching was done for 4 biological replicates and representative plates are shown. The underlined patches were selected for testing for the single colony streaks shown in Fig 6. The previously demonstrated positive RsbV (V)/RsbW (W) interactions[26] (indicated with # and detailed at the bottom of the figure) was used as an extra control in addition to the regular positive and negative BACTH controls (see main text for details).

Fig S11

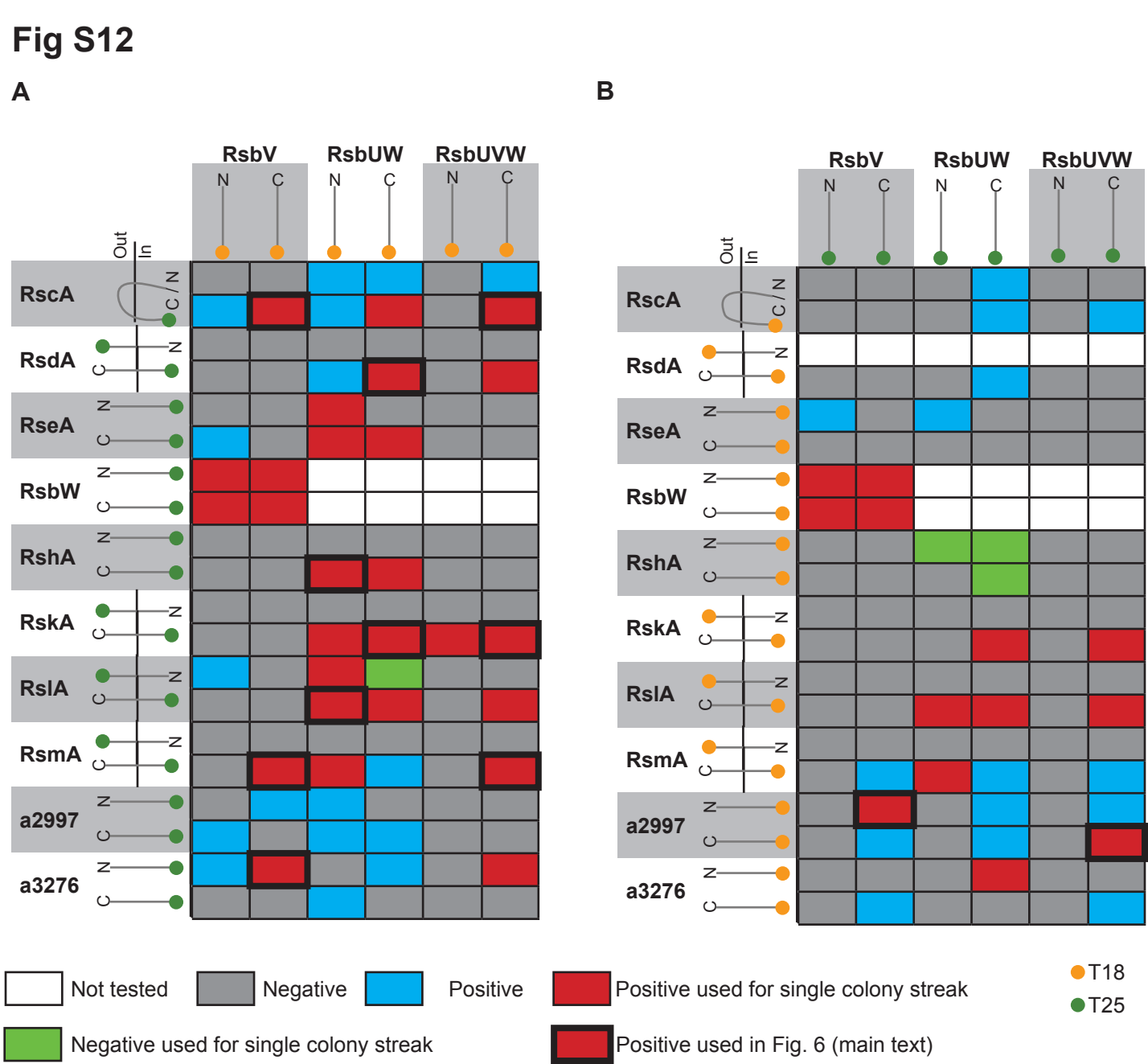


#	pKT25				pKNT25			
	W	V	W	V	W	V	W	V
	V	W	V	W	V	W	V	W
	pUT18		pUT18C		pUT18		pUT18C	

Figure S12 Summary of all tested anti-σ factor interactions with stressosome pathway proteins.

The stressosome pathway related proteins RsbUW and RsbUVW, the anti- anti-σ factor RsbV (to RsbW) and the anti-σ factors were cloned in the BACTH system and co-expressed in *E. coli* BTH101 cells and patched on LA + X-gal plates containing the appropriate antibiotics. If the patches had a blue color it was interpreted as being a positive interaction. The interactions were scored as positive, "+", weak or doubtful positive, "(+)", or negative. Interactions which had at least 3 "+" or "(+)" were considered as positive in the overall summary shown in the figure as red or green boxes. Patching was done for four biological replicates (of which one representative replicate is shown in Fig S2) and a representative summary is shown. Colonies representing the red boxes (also positive) and the green boxes (negative) were selected for testing for single colony streaks and the red boxes with thick borders represent the colonies shown in Fig 6 (see main text). The cartoons indicate a simplified structure and membrane topology of the fusion proteins where N and C are the free N- and C-termini of the σ factors and anti-σ factors (grey lines), and the green and orange dots are the adenylate cyclase T25 and T18 domains, respectively, which need to interact to activate β-galactosidase expression. The black line represents the cytoplasmic membrane and the in- and outside is indicated. See also figure legends S2 and S5.

Fig S12


A
B
RsbV
RsbUW
RsbUVW
N
C
Out
In
RscA
RsdA
RseA
RsbW
RshA
RskA
RslA
RsmA
a2997
a3276
Not tested
Negative
Positive
Positive used for single colony streak
Negative used for single colony streak
Positive used in Fig. 6 (main text)
T18
T25

406 **Figure S13 Multiple sequence alignment.**

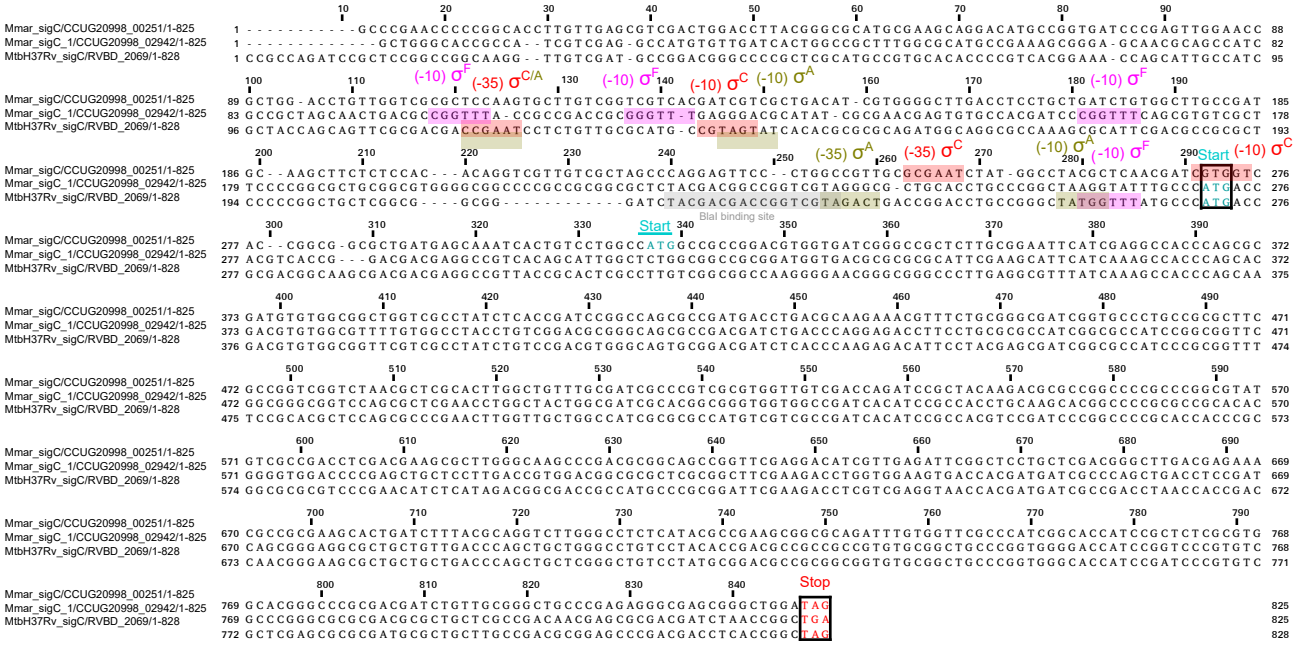
407 Multiple sequence alignment of: **A** Sigma factor *sigC*/*sigC*_1 present in, and **B** anti-sigma
408 factor *rscA* in *M. marinum* CCUG20998 and *M. tuberculosis* H37Rv as indicated. The putative
409 *sigC*, *sigC*_1 and *rscA* promoters as indicated. With respect to the *M. tuberculosis* H37Rv *sigC*
410 promoters see[54]. The predicted translation start and stop codons are marked in turquoise and
411 red, respectively.

412

413

Fig S13

A


Mmar_sigC/CCUG20998_00251/1-825
Mmar_sigC_1/CCUG20998_02942/1-825
MtbH37Rv_sigC/RVBD_2069/1-828
(-10) σF
(-35) σC/A
(-10) σF
(-10) σC
(-10) σA
(-35) σA
(-35) σC
(-10) σA
(-10) σF
Start
(-10) σC
Blal binding site
Start
Stop

B

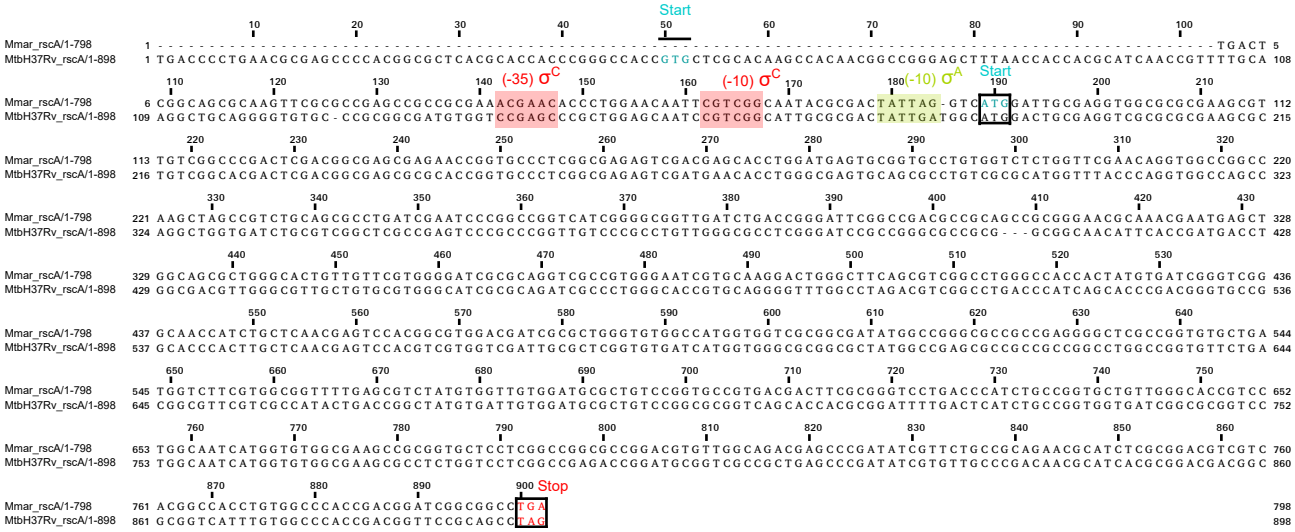

Start
Mmar_rscA/1-798
MtbH37Rv_rscA/1-898
(-35) σC
(-10) σC
(-10) σA
Start
Stop

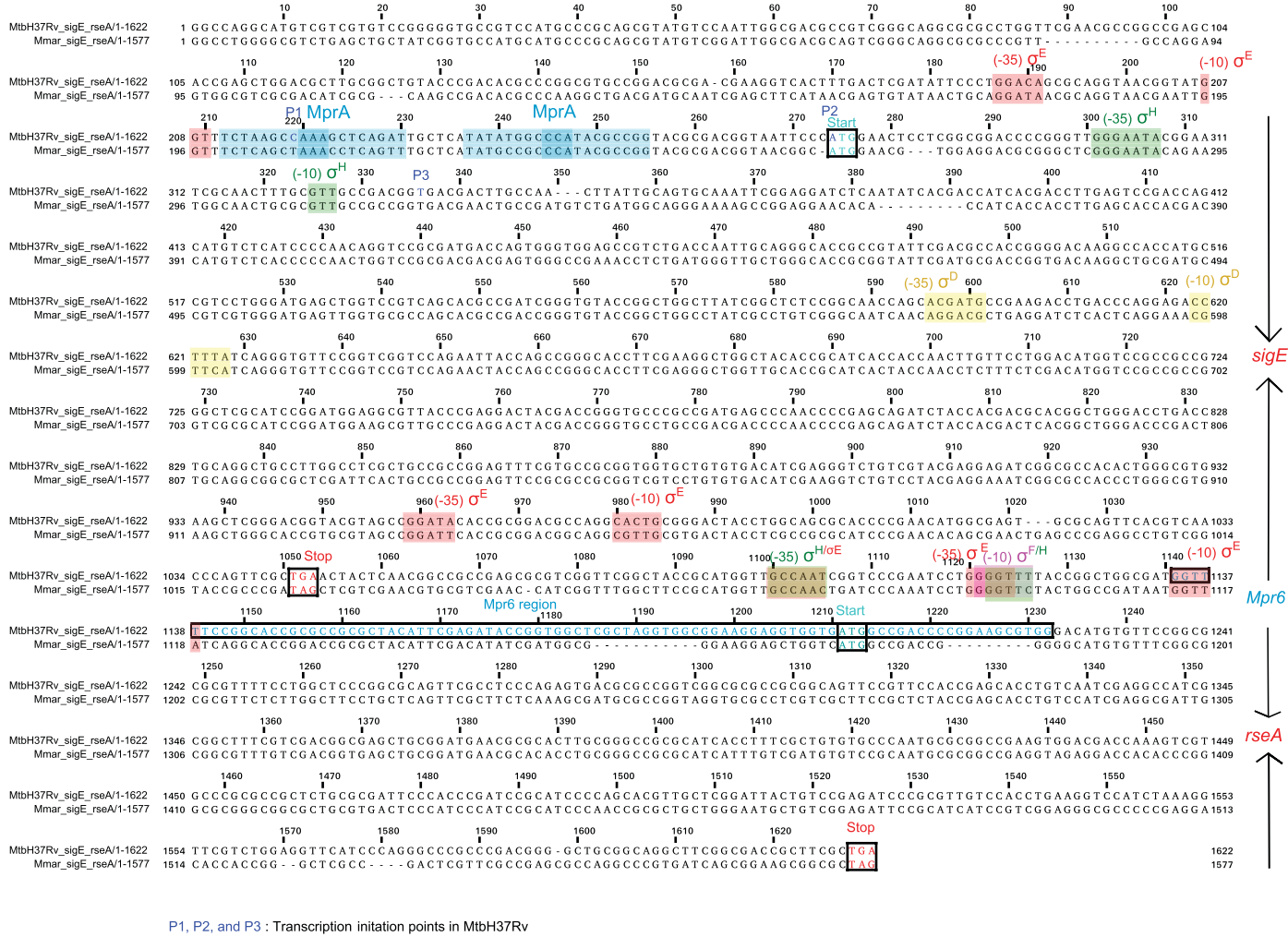
Figure S14 Sequence information and mRNA transcript levels for the genes *sigE*, *mpr6*, and *rseA*.

A Multiple sequence alignment of the genomic region between *sigE* and anti-SigE (*rseA*) in *M. marinum* CCUG20998 and *M. tuberculosis* H37Rv. Putative promoter sequences (-35 and -10 boxes) are indicated with background shading. The Mpr6 RNA identified in *M. tuberculosis* H37Rv is indicated in blue and boxed, while blue background shading indicates putative MprA binding sites. The three transcription start sites identified in *M. tuberculosis* H37Rv are indicated in dark blue and marked P1 to P3. Boxed translational start and stop codons indicate the beginning and ending of the *sigE* and *rseA* coding sequences.

B Bar plots showing mRNA transcript levels for *sigE*, *mpr6*, and *rseA* in *M. marinum* CCUG20998 in response to various stress and growth condition. Statistical significance: P-values are represented with asterisks, *p < 0.05; **p < 0.01; ***p < 0.001.

Fig S14

A



P1, P2, and P3 : Transcription initation points in MtbH37Rv

B

