A portable regulatory RNA array design enables tunable and complex regulation across diverse bacteria

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Supplementary Table 1. All plasmids used in this study. CmR: chloramphenicol resistance gene, AmpR: ampicillin resistance gene, SpcR: spectinomycin resistance gene, KanR: kanamycin resistance gene, p15A: origin of replication, ColE1: origin of replication, CDF: CloDF origin of replication, pBBR1: broad-host range origin of replication, pSP6: SP6 promoter, TrrnB: terminator, t500: terminator, RBS: ribosome binding site, Csy4: Csy4 ribonuclease, csy4hp: csy4 cleavage hairpin, shcsyhp: strong hairpin csy4 cleavage hairpin, PlmJ: ribozyme.

Plasmid	Description	Plasmid Architect	Figure
pBL176	STAR positive	J23119-Target10-RBS-GFP-TrrnB-J23119-STAR10-t500-pBBR1-KanR	Fig1
pBL177	STAR negative	J23119-Target10-RBS-GFP-TrrnB-pBBR1-KanR	Fig1
pBL301	STAR negative+Csy4	J23119-Target10-RBS-GFP-TrrnB-t500-J23101-Csy4-Terminator-pBBR1-KanR	Fig1/Fig4
pBL302	STAR positive+Csy4	J23119-Target10-RBS-GFP-TrrnB-J23119-STAR10-t500-J23101-Csy4-Terminator-pBBR1-KanR	Fig1
pBL115	SP6 RNAP	AraC-pBAD-RBS-SP6 RNAP-TrrnB-SpcR-CDF	Fig1/FigS1
pBL068	SP6 Target-GFP	pSP6-Target6-RBS-GFP-TrrnB-CmR-p15A	Fig1
pBL071	SP6 STAR	pSP6-STAR6-t500-ColE1-AmpR	Fig1
pJEC102	Control	J23119-TrrnB-ColE1-AmpR	Fig1/FigS1
pJEC250	Target50-GFP	J23119-Target50-RBS-GFP-TrrnB-CmR-p15A	Fig2/FigS3
pBLM443	PlmJ_STAR50x4	AraC_pBAD-PlmJ_STAR50x4-t500-ColE1-AmpR	Fig2
pBLM444	PlmJ_STAR50x1	AraC_pBAD-PlmJ_STAR50x1-t500-ColE1-AmpR	Fig2
pBL111	Csy4	J23101-Csy4-CDF-SpcR	Fig2
pBL451	csy4hp_STAR50x4	J23119-Target50-RBS-GFP-TrrnB-AraC_pBAD-csy4hp_STAR50x4-CmR-p15A	Fig2
pBL452	csy4hp_STAR50x1	J23119-Target50-RBS-GFP-TrrnB-AraC_pBAD-csy4hp_STAR50x1-CmR-p15A	Fig2
pBL170	shcsy4hp_STAR50x4	J23119-Target50-RBS-GFP-TrrnB-AraC_pBAD-shcsy4hp_STAR50x4-CmR-p15A	Fig2
pBL171	shcsy4hp_STAR50x1	J23119-Target50-RBS-GFP-TrrnB-AraC_pBAD-shcsy4hp_STAR50x1-CmR-p15A	Fig2

pBL161	shcsy4hp_STAR10x4	J23119-Target10-RBS-GFP-TrrnB-AraC_pBAD-shcsy4hp_STAR10x4-CmR-p15A	Fig2
pBL162	shcsy4hp_STAR10x1	J23119-Target10-RBS-GFP-TrrnB-AraC_pBAD-shcsy4hp_STAR10x1-CmR-p15A	Fig2
pJEC216	Target10-GFP	J23119-Target10-RBS-GFP-TrrnB-CmR-p15A	Fig2/Fig4/FigS3
pBL303	STAR10x1 (pBAD)	J23119-Target10-RBS-GFP-TrrnB-AraC_pBAD-shcsy4hp_STAR10x1-J23101-Csy4-Terminator-pBBR1-KanR	Fig3/FigS6
pBL304	STAR10x2 (pBAD)	J23119-Target10-RBS-GFP-TrrnB-AraC_pBAD-shcsy4hp_STAR10x2-J23101-Csy4-Terminator-pBBR1-KanR	Fig3/FigS6
pBL305	STAR10x4 (pBAD)	J23119-Target10-RBS-GFP-TrrnB-AraC_pBAD-shcsy4hp_STAR10x4-J23101-Csy4-Terminator-pBBR1-KanR	Fig3/FigS6
pBL306	STAR10x6 (pBAD)	J23119-Target10-RBS-GFP-TrrnB-AraC_pBAD-shcsy4hp_STAR10x6-J23101-Csy4-Terminator-pBBR1-KanR	Fig3/FigS6
pBL307	STAR10x8 (pBAD)	J23119-Target10-RBS-GFP-TrrnB-AraC_pBAD-shcsy4hp_STAR10x8-J23101-Csy4-Terminator-pBBR1-KanR	Fig3/FigS6
pBL401	STAR10x1 (pCymR)	J23119-Target10-RBS-GFP-TrrnB-CymR_pCymR-shcsy4hp_STAR10x1-J23101-Csy4-Terminator-pBBR1-KanR	Fig3/FigS6
pBL402	STAR10x2 (pCymR)	J23119-Target10-RBS-GFP-TrrnB-CymR_pCymR-shcsy4hp_STAR10x2-J23101-Csy4-Terminator-pBBR1-KanR	Fig3/FigS6
pBL403	STAR10x4 (pCymR)	J23119-Target10-RBS-GFP-TrrnB-CymR_pCymR-shcsy4hp_STAR10x4-J23101-Csy4-Terminator-pBBR1-KanR	Fig3/FigS6
pBL404	STAR10x6 (pCymR)	J23119-Target10-RBS-GFP-TrrnB-CymR_pCymR-shcsy4hp_STAR10x6-J23101-Csy4-Terminator-pBBR1-KanR	Fig3/FigS6
pBL405	STAR10x8 (pCymR)	J23119-Target10-RBS-GFP-TrrnB-CymR_pCymR-shcsy4hp_STAR10x8-J23101-Csy4-Terminator-pBBR1-KanR	Fig3/FigS6
pBL512	pBAD-GFP	AraC_pBAD-RBS-GFP-TrrnB-pBBR1-KanR	Fig3/FigS5

pBL513	pCymR-GFP	CymR_pCymR-RBS-GFP-TrrnB-pBBR1-KanR	Fig3/FigS5
pBLM366	Target50-STAR10x1	J23119-Target50-shCsy4_STAR10x1-t500-RSF1030-SpcR	Fig4
pBLM367	Target50-STAR10x4	J23119-Target50-shCsy4_STAR10x4-t500-RSF1030-SpcR	Fig4
pJEC251	STAR50	J23119-STAR50-t500-ColE1-AmpR	Fig4
pBL501	Target10-GFP+Target50-STAR10x1	J23119-Target10-RBS-GFP-TrrnB-J23119-Target50-shCsy4_STAR10x1-t500-J23101-Csy4-Terminator-pBBR1-KanR	Fig4
pBL502	Target10-GFP+Target50-STAR10x4	J23119-Target10-RBS-GFP-TrrnB-J23119-Target50-shCsy4_STAR10x4-t500-J23101-Csy4-Terminator-pBBR1-KanR	Fig4
pBL504	Target10-GFP+Target50-STAR10x1 +STAR50	J23119-Target10-RBS-GFP-TrrnB-J23119-Target50-shCsy4_STAR10x1-t500-J23119-STAR50-t500-J23101-Csy4-Terminator-pBBR1-KanR	Fig4
pBL505	Target10-GFP+Target50-STAR10x4 +STAR50	J23119-Target10-RBS-GFP-TrrnB-J23119-Target50-shCsy4_STAR10x4-t500-J23119-STAR50-t500-J23101-Csy4-Terminator-pBBR1-KanR	Fig4
pBL506	Target10-GFP+Target50-mRFP+STAR10x1 +STAR50x1	J23119-Target10-RBS-GFP-TrrnB-J23119-RBS-RFP-t500-AraC_pBAD-shcsy4hp_STAR10x1-shcsy4hp_STAR50x1-t500-J23101-Csy4-Terminator-pBBR1-KanR	Fig4/FigS8
pBL507	Target10-GFP+Target50-mRFP+STAR10x1 +STAR50x2	J23119-Target10-RBS-GFP-TrrnB-J23119-RBS-RFP-t500-AraC_pBAD-shcsy4hp_STAR10x1-shcsy4hp_STAR50x2-t500-J23101-Csy4-Terminator-pBBR1-KanR	Fig4/FigS8
pBL508	Target10-GFP+Target50-mRFP+STAR10x2 +STAR50x1	J23119-Target10-RBS-GFP-TrrnB-J23119-RBS-RFP-t500-AraC_pBAD-shcsy4hp_STAR10x2-shcsy4hp_STAR50x1-t500-J23101-Csy4-Terminator-pBBR1-KanR	Fig4/FigS8
pBL509	Target10-GFP+Target50-mRFP+STAR10x2 +STAR50x2	J23119-Target10-RBS-GFP-TrrnB-J23119-RBS-RFP-t500-AraC_pBAD-shcsy4hp_STAR10x2-shcsy4hp_STAR50x2-t500-J23101-Csy4-Terminator-pBBR1-KanR	Fig4/FigS8
pBL059	SP6 RNAP	pTet-RBS-luxR-Terminator-pLuxR-RBS-SP6 RNAP-TrrnB-SpcR-CDF	FigS1

pBL062	SP6 promoter-Target28-GFP	pSP6-Target28(AD1.S7)-RBS-sfGFP-TrrnB-CmR-p15A	FigS1
pJEC154	STAR28(12nt)	J23119-STAR28(12nt)-t500-ColE1-AmpR	FigS1
pJEC163	STAR28(17nt)	J23119-STAR28(17nt)-t500-ColE1-AmpR	FigS1
pJEC164	STAR28(27nt)	J23119-STAR28(27nt)-t500-ColE1-AmpR	FigS1
pJEC165	STAR28(37nt)	J23119-STAR28(37nt)-t500-ColE1-AmpR	FigS1
pJEC166	STAR28(47nt)	J23119-STAR28(47nt)-t500-ColE1-AmpR	FigS1
pJEC167	STAR28(57nt)	J23119-STAR28(57nt)-t500-ColE1-AmpR	FigS1
pBL116	pSP6(wt)-Target10-GFP	pSP6-Target10-RBS-GFP-TrrnB-CmR-p15A	FigS1
pBL120	pSP6(wt)-STAR10	pSP6-STAR10-t500-ColE1-AmpR	FigS1
pBL144	pSP6(90p)-STAR10	pSP6(90p)-STAR10-t500-ColE1-AmpR	FigS1
pBL145	pSP6(50p)-STAR10	pSP6(50p)-STAR10-t500-ColE1-AmpR	FigS1
pBL146	pSP6(20p)-STAR10	pSP6(20p)-STAR10-t500-ColE1-AmpR	FigS1
pBL117	pSP6-Target28-GFP	pSP6-Target28-RBS-GFP-TrrnB-CmR-p15A	FigS1
pBL118	pSP6-Target50-GFP	pSP6-Target50-RBS-GFP-TrrnB-CmR-p15A	FigS1
pBL121	pSP6-STAR28	pSP6-STAR28-t500-ColE1-AmpR	FigS1
pBL122	pSP6-STAR50	pSP6-STAR50-t500-ColE1-AmpR	FigS1

Supplementary Table 2. Example DNA plasmid sequence.

Name	DNA sequence
Regulatory RNA array plasmid STAR10x8 (pBL307) J23119-Target10-RBS-GFP-TrrnB-AraC-pBAD-(shcsy4hp_STAR10)x8-Csy4-pBBR1-KanR	GAATTCTAAAGATCTTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGT CTCATCTCATTTCGCTCTCACATTTCTACACCTTTATCTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTTGGATCTAGGAGGAAG GATCTATGAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTT GTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCCGTGGAGA GGGTGAAGGTGATGCTACAAACGGAAAACTCACCCTTAAATTTATTTGCA CTACTGGAAAACTACCTGTTCCGTGGCCAACACTTGTCACTACTCTGACC TATGGTGTTCAATGCTTTTCCCGTTATCCGGATCACATGAAACGGCATGA CTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATAT CTTTCAAAGATGACGGGACCTACAAGACGCGTGCTGAAGTCAAGTTTGA AGGTGATACCCTTGTTAATCGTATCGAGTTAAAGGGTATTGATTTTAAAG AAGATGGAAACATTCTTGGACACAAACTCGAGTACAACTTTAACTCACAC AATGTATACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTT CAAAATTCGCCACAACGTTGAAGATGGTTCCGTTCAACTAGCAGACCATT ATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAAC CATTACCTGTCGACACAATCTGTCCTTTCGAAAGATCCCAACGAAAAGCG TGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATG GCATGGATGAGCTCTACAAATAAGGATCTGAAGCTTGGGCCCGAACAAA AACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCAT CATCATTGAGTTTAAACGGTCTCCAGCTTGGCTGTTTTGGCGGATGAGA GAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTG ATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGACC CCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGG GGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAA AGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTG AACTGGATCCCCAATTATTGAAGGCCGCTAACGCGGCCTTTTTTTGTTTC TGGTCTGCCTTAATCAATGACTAAGAATTCGCGGCCGCTTCTAGAGCGT CTCACTTCGGATCCGCTGCAGGCTTCTCAAGTCAAAAGCCTCCGGTCGG AGGTTTTTGACTTTCTGCTATGGAGGTCAGGTATGATTTTTATGACAACTT GACGGCTACATCATTCACTTTTTCTTCACAACCGGCACGGAACTCGCTC GGGCTGGCCCCGGTGCATTTTTTAAATACCCGCGAGAAATAGAGTTGAT CGTCAAAACCAACATTGCGACCGACGGTGGCGATAGGCATCCGGGTGG TGCTCAAAAGCAGCTTCGCCTGGCTGATACGTTGGTCCTCGCGCCAGCT TAAGACGCTAATCCCTAACTGCTGGCGGAAAAGATGTGACAGACGCGAC GGCGACAAGCAAACATGCTGTGCGACGCTGGCGATATCAAAATTGCTGT CTGCCAGGTGATCGCTGATGTACTGACAAGCCTCGCGTACCCGATTATC CATCGGTGGATGGAGCGACTCGTTAATCGCTTCCATGCGCCGCAGTAAC AATTGCTCAAGCAGATTTATCGCCAGCAGCTCCGAATAGCGCCCTTCCC CTTGCCCGGCGTTAATGATTTGCCCAAACAGGTCGCTGAAATGCGGCTG GTGCGCTTCATCCGGGCGAAAGAACCCCGTATTGGCAAATATTGACGGC

CAGTTAAGCCATTCATGCCAGTAGGCGCGCGGACGAAAGTAAACCCACT
GGTGATACCATTCGCGAGCCTCCGGATGACGACCGTAGTGATGAATCTC
TCCTGGCGGGAACAGCAAAATATCACCCGGTCGGCAAACAAATTCTCGT
CCCTGATTTTTCACCACCCCCTGACCGCGAATGGTGAGATTGAGAATATA
ACCTTTCATTCCCAGCGGTCGGTCGATAAAAAAATCGAGATAACCGTTG
GCCTCAATCGGCGTTAAACCCGCCACCAGATGGGCATTAAACGAGTATC
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CGTTTTTTTGGGCTAGCCGACCTGTGGACGGATAAAGTCAGTTGTGAAA
TAAACGCGGCCATAGGCCGCGTAGTTCACTGCCGTATAGGCAGCTAAGA
AAAATGTGAACTGTATACATTCCCCGCAAGATAAAGGTGTAGAAATGTGA
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AAATGTGAGAGCGAAATGAGATGAGTGGCCAAAGCCCGCCGAAAGGCG
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TTAAGGCAGTTATTGGTGCCCTTAAACGCGTTCTTTACAGCTAGCTCAGT
CCTAGGTATTATGCTAGCAGATCTAAAGAGGAGAAAGGATCTATGGACC
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GCTCCCGGCTGGGCGAGCGCCTGCGCATTCATGCCTCGGCGGACGAC
CTTCGTGCCCTGCTCGCCCGGCCCTGGCTGGAAGGGTTGCGGGACCAT
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CAGGTCAGTCGGGTTCAGGCGAAAAGCAATCCGGAACGCCTGCGGCGG
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GCAAAGAACGTCCGATGAGCTTGGAAAGTGTCTTCTGGCTGACCACCAC
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CTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCG
CATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGG
GGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTT
CGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTC
CGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAG
TTCTTCGCCCACCCCCATGGGCAAATATTATACGCAAGGCGACAAGGTG
CTGATGCCGCTGGCGATTCAGGTTCATCATGCCGTTTGTGATGGCTTCC
ATGTCGGCAGAATGCTTAATGAATTACAACAGTTTTTATGCATGCGCCCA
ATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCT
GGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAA
TTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCCTAGATTTCAGTG
CAATTTATCTCTTCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAA
GTTGTAATTCTCATGTTTGACAGCTTATCATCGATAAGCTTCCGATGGCG
CGCCGAGAGGCTTTACACTTTATGCTTCCGGCT

Supplementary Table 3. SP6 promoter sequences used in this study.

Promoter	Sequence (5’ to 3’)
pSP6_wt	ATTTAGGTGACACTATAGGG
pSP6_90	ATTTAGGTGCCACTATAGGG
pSP6_50	ATTTAGGTGACACTATGGGG
pSP6_20	ATTTAGGTGACATTATAGGG

Supplementary Table 4. Golden Gate assembly primers and overhangs. An example of the 8-copy RNA array assembly. BsmBI site: GAGACG, BsaI site: GGTCTC, Overhang: ctcg. Table corresponds to the naming conventions shown in Supplementary Figure 2. We note that some of the parts indicated in the table below (e.g., spacer, connectors) are not included in Supplementary Figure 2 for simplicity. The top section of the table indicates how to construct a 4-copy STAR array modular cloning (MoClo) part for position 3b. Position 4 can be used to create another 4-copy STAR array MoClo part using the same overhang logic indicated in the top section of the table. If constructing a multiplex array (Figure 4D) the STAR identity in positions 3b and 4 can be varied.

Assembly parts	**Sequence (5' to 3')**
Level 1 => Level 2 (Position 3b)	
Backbone	- CGTCTCaaGAGACC - backbone - GGTCTCgtGAGACG - GFP dropout -
PCR products 1	gtCGTCTCactcgtcag - Insulator+STAR - cgtttGAGACGtg
PCR products 2	gtCGTCTCacgtt - Insulator+STAR - ttcctGAGACGtg
PCR products 3	gtCGTCTCattcc - Insulator+STAR - tcattGAGACGtg
PCR products 4	gtCGTCTCatcat - Insulator+STAR - aatgagagtGAGACGtg
Level 2 => Level 3	
Position 1	GGTCTCgacgg - Connector* - cttcaGAGACC
Position 2	GGTCTCgcttc - Promoter - cgcaaGAGACC
Position 3a	GGTCTCgcgca - Spacer/Target - tcagaGAGACC
Position 3b	GGTCTCgtcag - Inserts - aatgaGAGACC
Position 4	GGTCTCgaatg - Inserts - tggcaGAGACC
Position 5	GGTCTCgtggc - Terminator - gctgaGAGACC
Position 6	GGTCTCggctg - Connector - tacaaGAGACC
Position 7	GGTCTCgtaca - Resistance & Origin of replication - acggaGAGACC

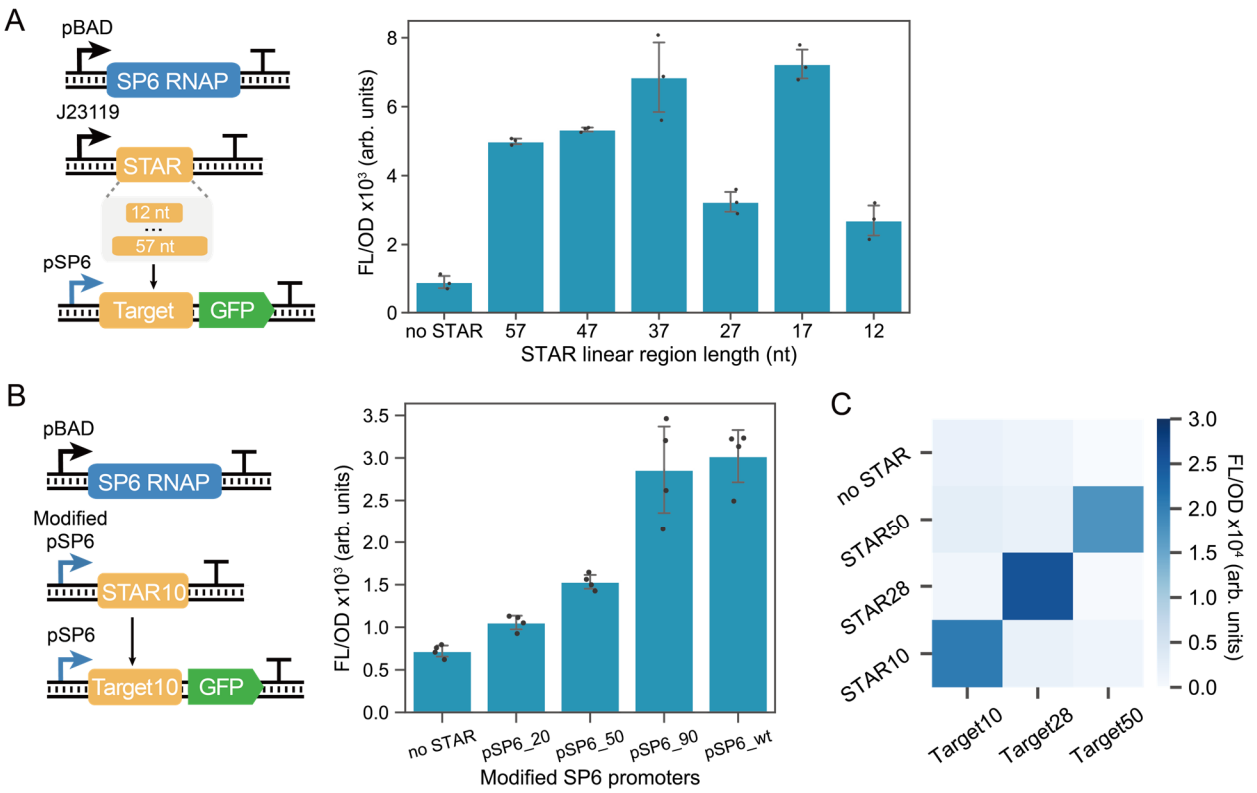
*Connectors contain BsmBI sites allowing further assembly

Supplementary Table 5. Strains, growth conditions, and electroporation settings used in this study.

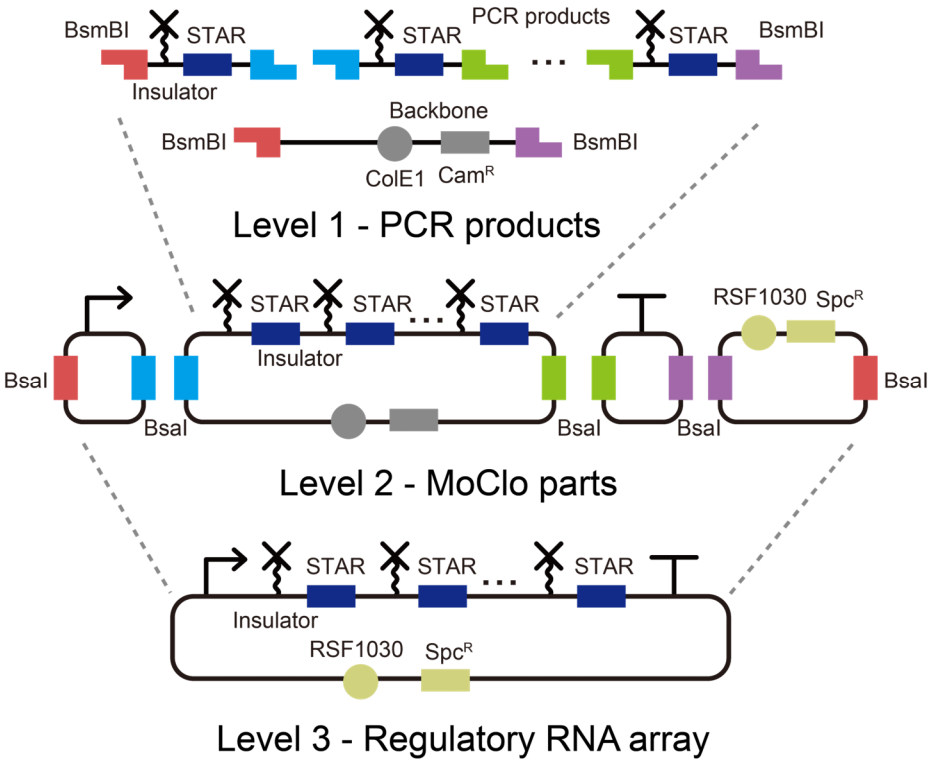
Species	Strain name	Culture media	Culture temperature (ºC)	Kanamycin conc. (µg/mL)	Electroporation setting (kV)
Escherichia coli	NEB Turbo (cloning strain)	LB	37	100	-
Escherichia coli	TG1	LB	37	100	-
Shewanella oneidensis	MR-1	LB	30	50	1.2
Pseudomonas fluorescens	A506	LB	30	50	2.5
Pseudomonas putida	F1	LB	30	50	1.25
Pseudomonas stutzeri	JM300	LB	30	50	2.5
Vibrio natriegens	Vmax	LB3	37	200	0.7

Supplementary Table 6. Regulatory RNA array insulator sequences.

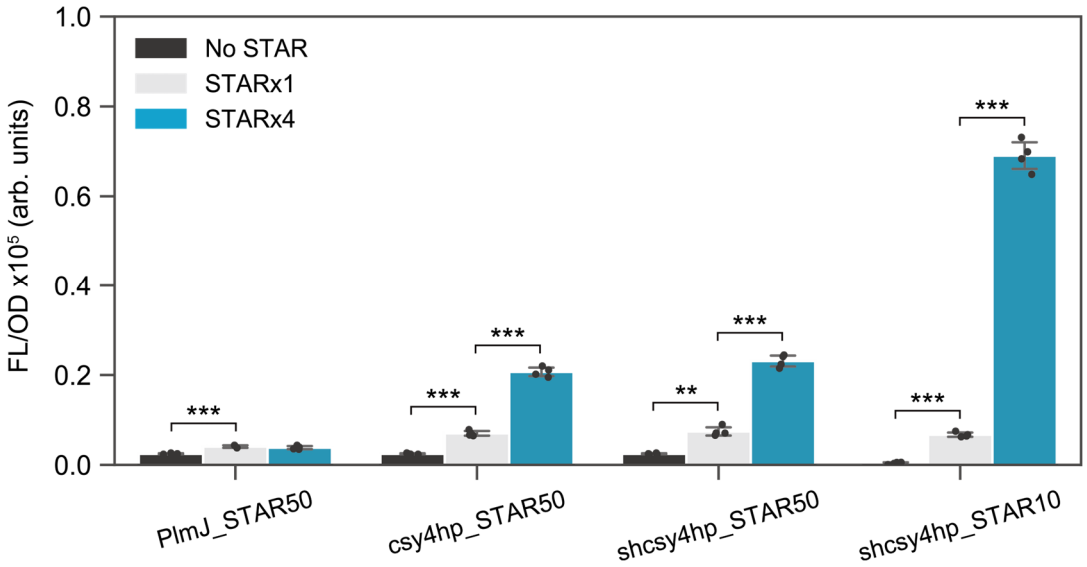
Insulator	Sequence (5' to 3')
PlmJ	AGUCAUAAGUCUGGGCUAAGCCCACUGAUGAGUCGCUGAAAUGCGACG AAACUUAUGA
csy4hp	GUUCACUGCCGUAUAGGCAGCUAAGAAA
shcsy4hp	UUGUGAAAUAAACGCGGCCAUAGGCCGCGUAGUUCACUGCCGUAUAGG CAGCUAAGAAA
PlmJ-SATR50x4 (Example sequence used for NUPACK simulation)	AGUCAUAAGUCUGGGCUAAGCCCACUGAUGAGUCGCUGAAAUGCGACG AAACUUAUGAUGAACUGUAUACAUUCCCCGCAAAGUGCCUAUCUGUCG UCGUGUUAUCUUUAUGUUUCUGGCGUUAGUCAUAAGUCUGGGCUAAG CCCACUGAUGAGUCGCUGAAAUGCGACGAAACUUAUGAUGAACUGUAU ACAUUCCCCGCAAAGUGCCUAUCUGUCGUCGUGUUAUCUUUAUGUUUC UGGUUCCAGUCAUAAGUCUGGGCUAAGCCCACUGAUGAGUCGCUGAAA UGCGACGAAACUUAUGAUGAACUGUAUACAUUCCCCGCAAAGUGCCUA UCUGUCGUCGUGUUAUCUUUAUGUUUCUGGUCAUAGUCAUAAGUCUG GGCUAAGCCCACUGAUGAGUCGCUGAAAUGCGACGAAACUUAUGAUGA ACUGUAUACAUUCCCCGCAAAGUGCCUAUCUGUCGUCGUGUUAUCUUU AUGUUUCUGG



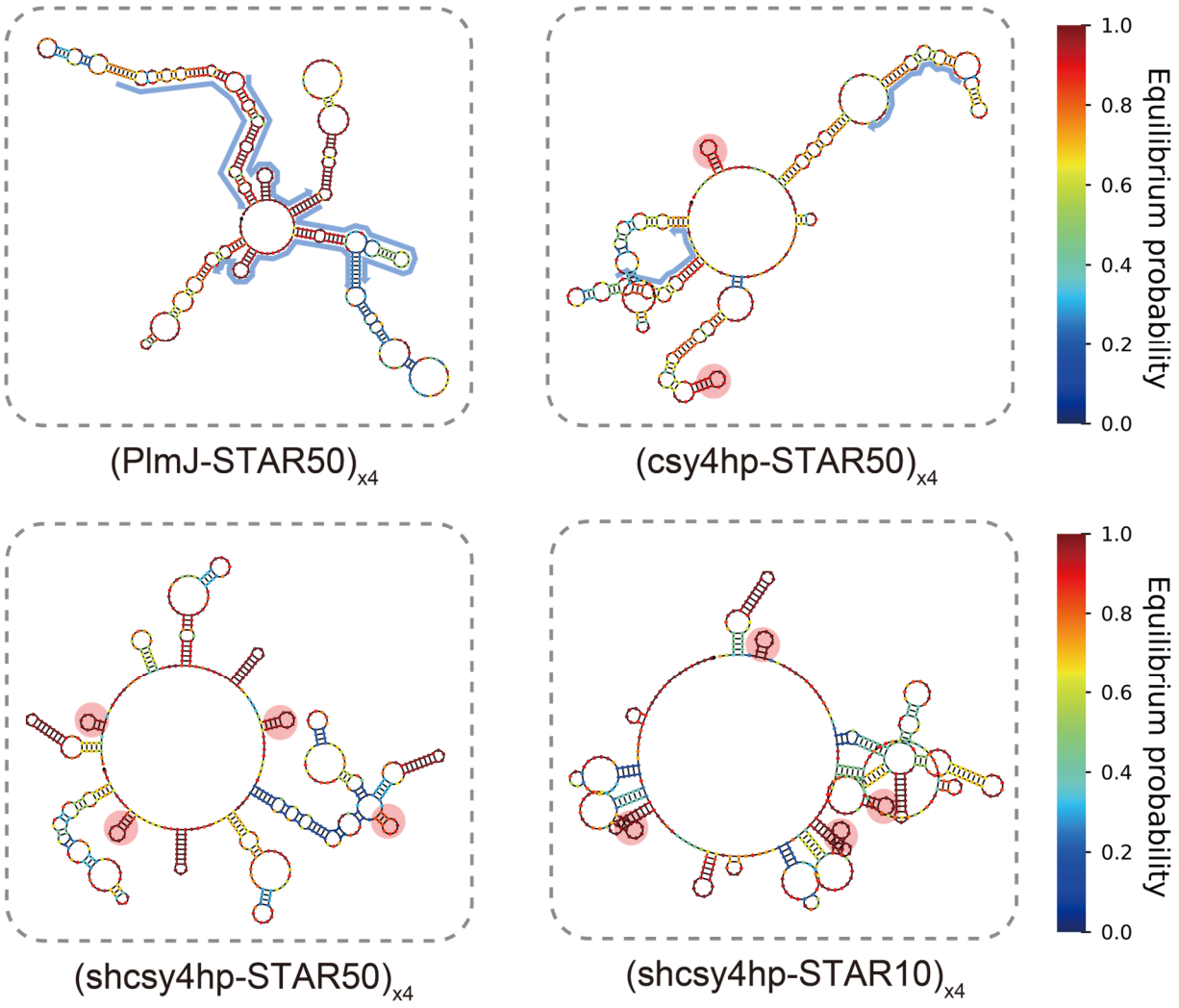
Supplementary Fig. 1. Characterization of a STAR system that uses the SP6 RNA polymerase. (A) Investigating the impact of length on the SP6 STAR system. STAR systems with different length linear regions (12 to 57 nucleotides [nt]) are used to activate a target RNA-controlled GFP that is transcribed using an SP6 promoter. The left panel shows a schematic of genetic circuitry and the right panel shows fluorescent characterization in *E. coli* cells transformed with corresponding plasmids. The results show a length of ~40 nt to be optimal which is comparable to prior STAR characterization results using the native *E. coli* RNAP[1]. Bars show mean values and error bars represent s.d. of $n = 3$ biological replicates shown as points. (B) Investigating the tunability of SP6-controlled STARs. STAR systems transcribed using only SP6 RNAP. The left panel shows a schematic of genetic circuitry and the right panel shows fluorescent characterization in *E. coli* cells transformed with corresponding plasmids. Variable strength SP6 promoters[2] are used to produce the STAR and a constant SP6 promoter is used to produce the target RNA. Bars show mean values and error bars represent s.d. of $n = 4$ biological replicates shown as points. (C) Investigating the orthogonality of SP6 STARs. Fluorescent characterization of cells transformed with different combinations of plasmids encoding cognate and non-cognate STAR and target RNA pairs. Cognate pairs are across the diagonal and the no STAR control is in the top row. Heatmap shows the mean values of $n = 4$ biological replicates.



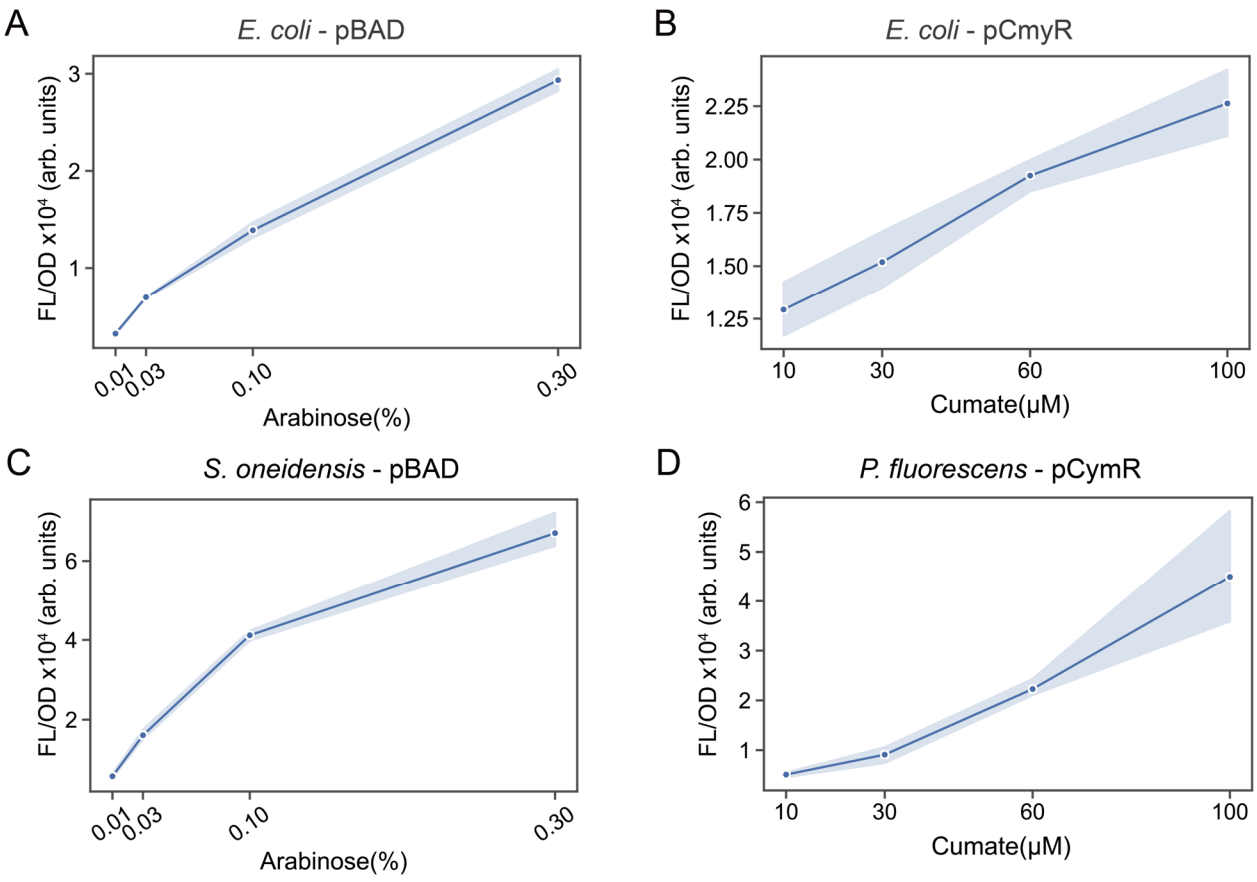
Supplementary Fig. 2. A modular cloning (MoClo) approach for regulatory RNA arrays. The regulatory RNA array is constructed through a 3-level modular cloning approach. PCR products are made with primers containing the recognition site for type 2 restriction enzyme BsmBI. The copy number of STARs can be altered by adjusting the number of PCR fragments with different overhangs. The PCR products are then assembled as MoClo part plasmids containing BsaI sites for long-term storage. Next, the MoClo parts are further assembled as the target plasmid with full regulatory RNA array cassette. We note that some of the parts indicated in Supplementary Table 4 (*e.g.,* spacer, connectors) are not included in this for simplicity.



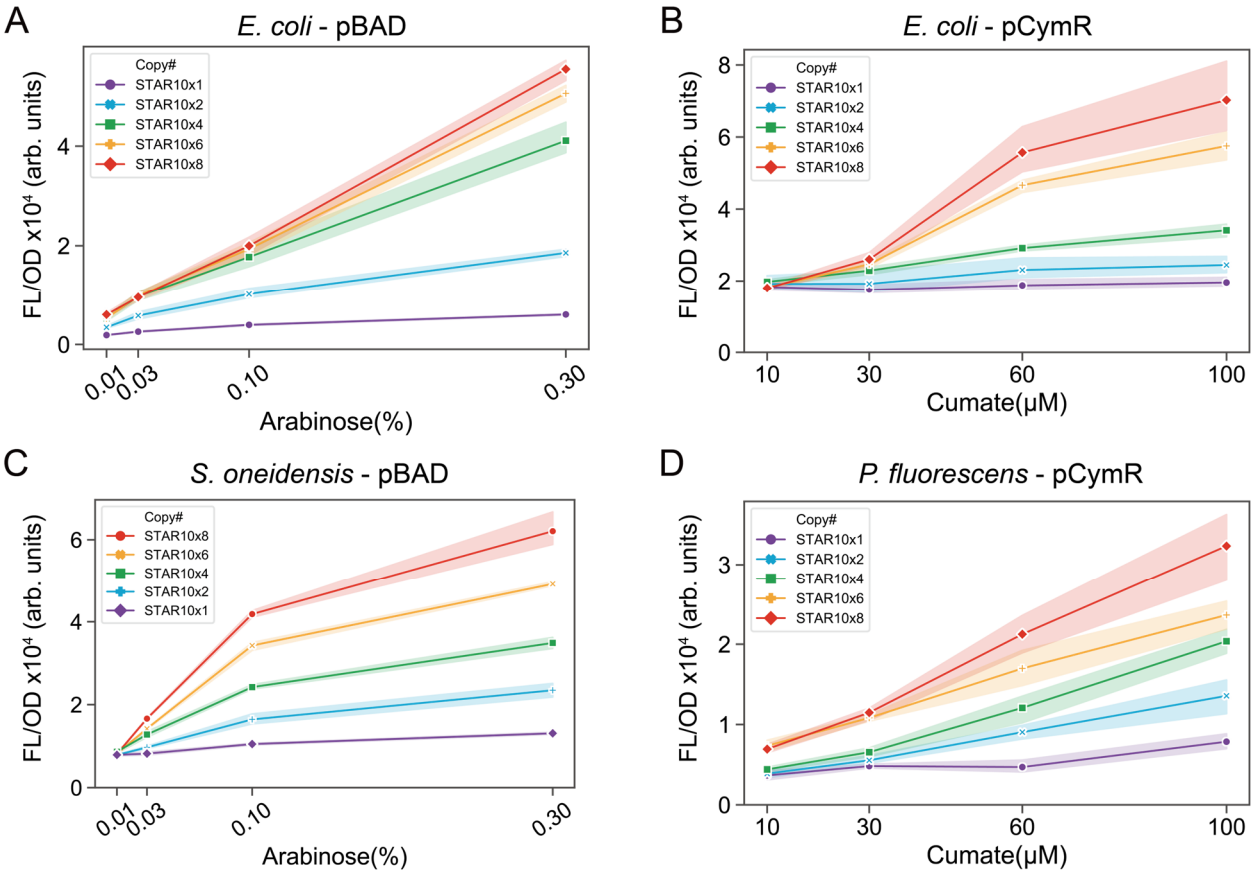
Supplementary Fig. 3. Insulator screening for regulatory RNA arrays. Fluorescent characterization of regulatory RNA arrays using PlmJ, csy4 hairpin, strong-hairpin csy4 hairpin as insulators. STAR50 and STAR10 arrays are tested with either x1, x4 or no copies. Bars show mean values and error bars represent s.d. of $n = 4$ biological replicates shown as points. Two-tailed t-tests assuming unequal variance were used and the significance are marked by asterisks indicating $p<.05$ (*), $p<.01$ (**), $p<.001$ (***).



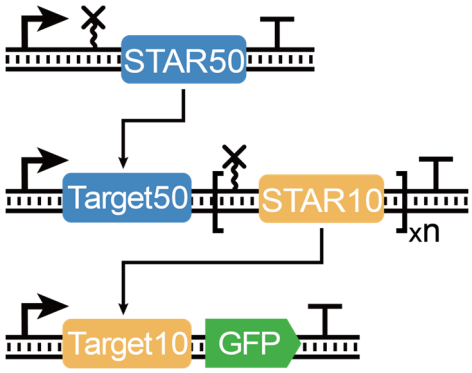
Supplementary Fig. 4. NUPACK analysis of regulatory RNA arrays with different insulators. Secondary structure predictions of 4-copy STAR arrays insulated with ribozyme PlmJ, ribonuclease site csy4 (csy4hp), and a strong-hairpin csy4 (shcsy4hp). Predictions use the online version of NUPACK[3]. The color of each nucleotide represents the equilibrium probability for each nucleotide to be in that state (e.g., paired/unpaired). Correctly folded insulator structures are labeled with red shading and insulator sequences that do not form the expected structure are indicated with blue shading.



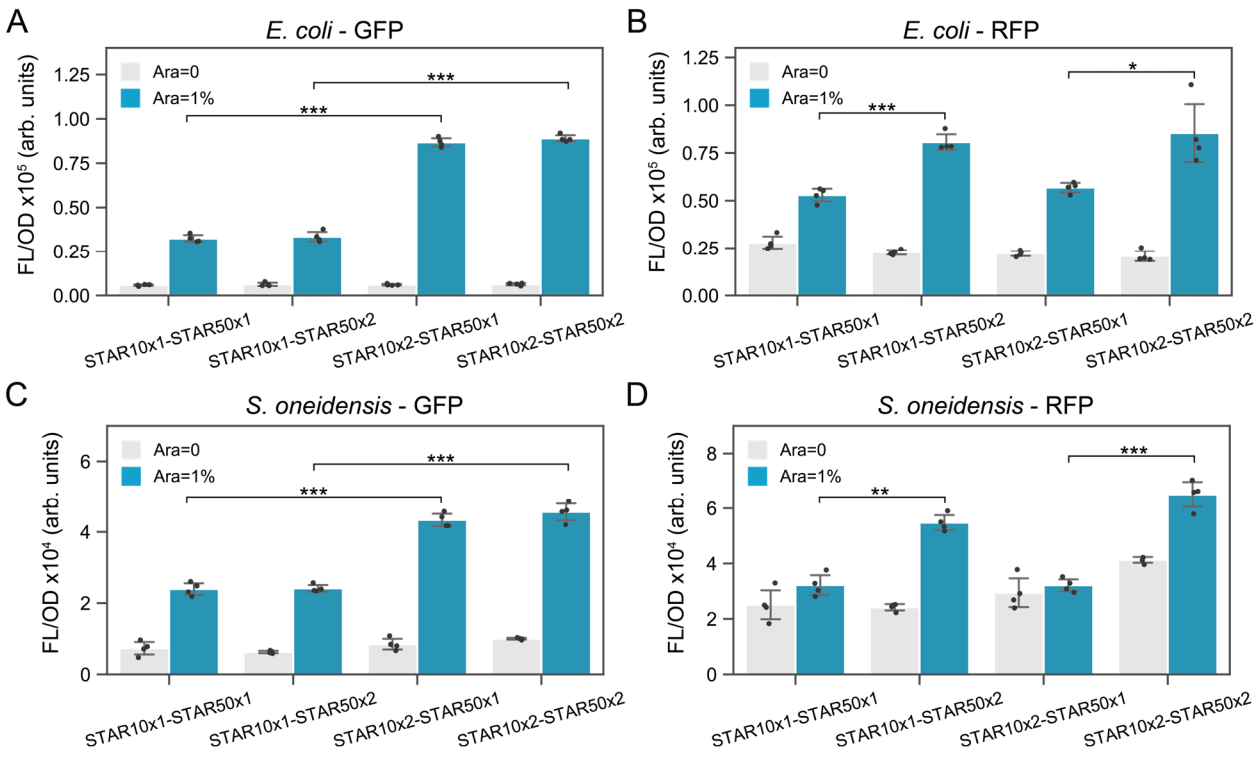
Supplementary Fig. 5. Calibration curves of inducible promoters to relate promoter strength to transcriptional output in different bacterial species. Fluorescent characterization of cells transformed with plasmids encoding a GFP driven by either pCymR or pBAD under different concentrations of inducer. Graphs show data from (A) pBAD in *E. coli*, (B) pCymR in *E. coli,* (C) pBAD in *S. oneidensis,* and (D) pCmyR in *P. fluorescens*. Each point shows the mean value and the shade represents s.d. of $n = 4$ biological replicates.



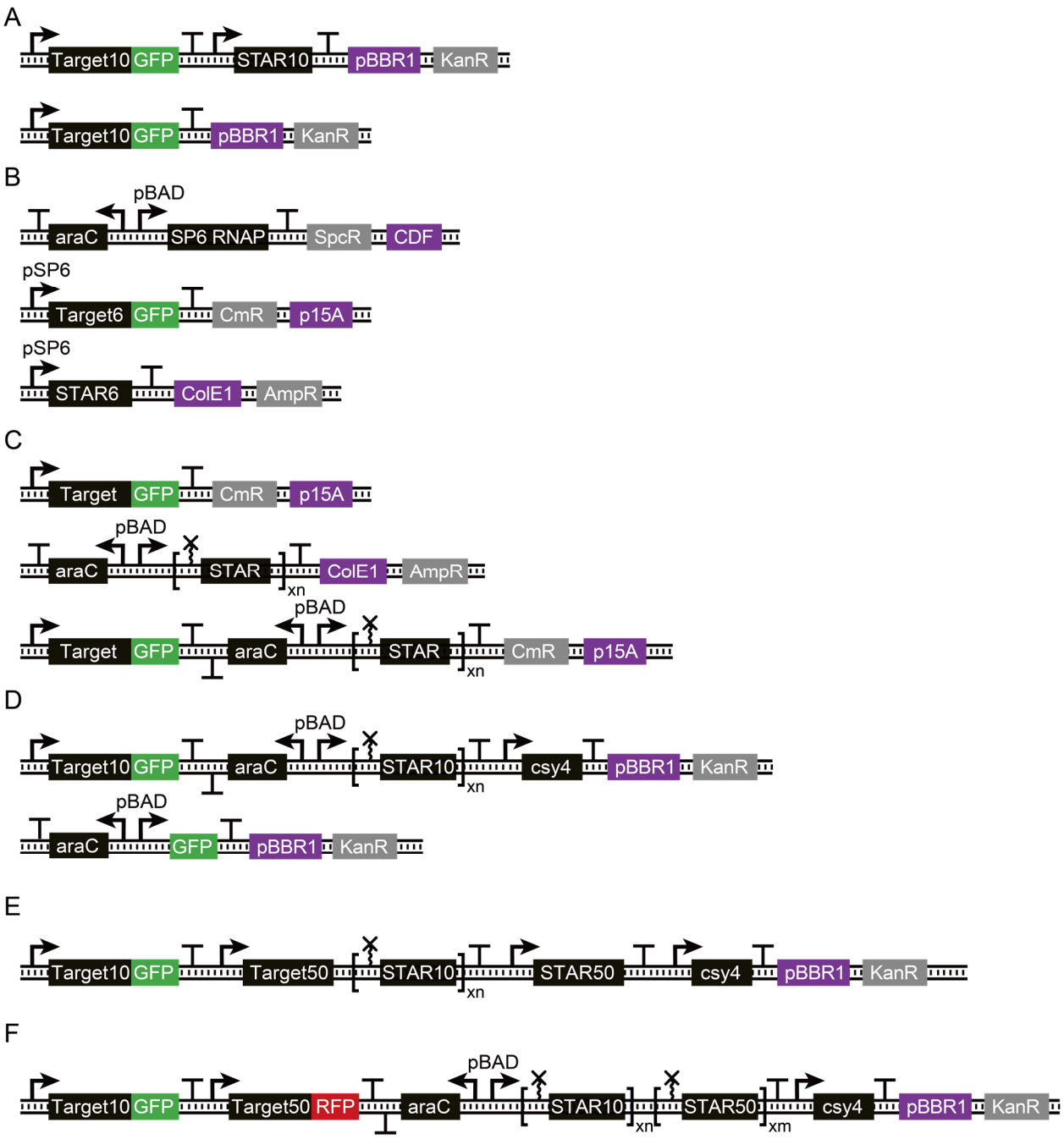
Supplementary Fig. 6. The induction curves of the regulatory RNA arrays before the promoter activity normalization. Fluorescent characterization of cells transformed with plasmids encoding regulatory RNA arrays with 1, 2, 4, 6, and 8 copies of STARs driven by either pCymR or pBAD under different concentrations of inducer. Graphs show data from (A) pBAD in *E. coli*, (B) pCymR in *E. coli,* (C) pBAD in *S. oneidensis,* and (D) pCmyR in *P. fluorescens*. Each point shows the mean value and the shade represents s.d. of $n = 4$ biological replicates.



Supplementary Fig. 7. The schematic of an RNA activation-activation cascade. The RNA cascade is built with an orthogonal pair STAR10 and STAR50. STAR50 is constitutively transcribed and activates the transcription of the regulatory RNA array composed of variable copies of STAR10. STAR10 production leads to the activation of Target10, which in turn activates the production of GFP.



Supplementary Fig. 8. Fluorescent characterization of multiplex RNA arrays. Fluorescent characterization of the multiplex RNA arrays with the 2x2 matrix of 4 possible STAR10 and STAR50 combinations. The graphs show uninduced and induced (arabinose=1%) data from (A) GFP characterization in *E. coli*, (B) RFP characterization in *E. coli*, (C) GFP characterization in *S. oneidensis*, (D) RFP characterization in *S. oneidensis*. Bars show mean values and error bars represent s.d. of $n = 4$ biological replicates shown as points. Two-tailed t-tests assuming unequal variance were used and the significance are marked by asterisks indicating $p<.05$ (*), $p<.01$ (**), $p<.001$ (***).



Supplementary Fig. 9. The schematics of representative plasmids used in this study. The example plasmid set used in (A) STAR for Gram-negative RNAP, (B) SP6 RNAP-driven STAR, (C) regulatory RNA array insulation screening, (D) regulatory RNA array and standard inducible GFP on broad host range plasmid, (E) single-plasmid RNA cascade, (F) multiplex regulatory RNA array.

Supplementary Note 1. Mathematical model of regulatory RNA arrays.
The model consists of two main process: (1) Csy4 processing of the RNA array and (2) the regulation of small transcription activating RNA (STAR) system. For the rest of this note, we will use capital letter to indicate chemical species, and lower case to denote the corresponding concentration. For example, species X has concentration x.

Modeling Csy4 cleaving. First, we define P^* to describe the unbound molecular specie Csy4, P for bound Csy4 to RNA array, and X_n for the full RNA array with n repeats. We consider a production rate constant α and θ for P^* and X_n respectively. A degradation rate constant δ and ϕ for P^* and X_n. n unbound proteins P^* can bind/unbinding to a single RNA array X_n and form a complex P with a rate constant k_p^+ and k_p^-. Finally, the state P can cleave and produce n copies of STAR X at a rate constant r_p, which decays at a rate constant ϕ. We summarize the reactions below:

$\emptyset \xrightarrow{\alpha} P^*$	$P^* \xrightarrow{\delta} \emptyset$	Production/Degradation
$\emptyset \xrightarrow{\theta} X_n$	$X_n \xrightarrow{\phi} \emptyset$	Production/Degradation
$X_n + nP^* \underset{\kappa_p^-}{\overset{\kappa_p^+}{\rightleftharpoons}} P$	$P \xrightarrow{r_p} nX.$	Binding/Unbinding/Cleaving
	$X \xrightarrow{\phi} \emptyset$	Degradation

As a result, we can write down the Ordinary Differential Equations (ODEs) by using the law of mass action:

$$\dot{p^*} = \alpha - \delta p^* - n * k_p^+ x_n p^* + n k_p^- p \quad (1)$$
$$\dot{x_n} = \theta - \phi x_n - k_p^+ x_n p^* + k_p^- p \quad (2)$$
$$\dot{p} = k_p^+ x_n p^* - k_p^- p - r_p p - \delta p \quad (3)$$
$$\dot{x} = n r_p p - \phi x \quad (4)$$

Modeling STAR system. A cleaved STAR X can bind to the target RNA complex C^* in which transcription is blocked and form a complex C to activate transcription, with a binding and unbinding rate k_c^+, and k_c^-. These interactions will modify the model for x, equation (4). The activated complex C then transcribes the output Y at the rate of r_c. Also, we consider leakiness when the blocked transcription complex C^* can produce an output species Y at a rate constant r_0. Finally, abortion can be incorporated when the activate transcription complex C become C^* at a rate constant ξ. We summarize the chemical reactions below:

$X + C^* \underset{\kappa_c^-}{\overset{\kappa_c^+}{\rightleftharpoons}} C$	$C \xrightarrow{r_c} C^* + Y.$	Activation
$Y \xrightarrow{\phi} \emptyset$		Decay
$C^* \xrightarrow{r_0} Y$	$C \xrightarrow{\xi} C^*$	Leakiness/Abortion

Following law mass of action, we can find the ODEs:

$$\dot{x} = n r_p p - \phi x - \kappa_c^+ x c^* + \kappa_c^- c \quad (5)$$
$$\dot{c} = \kappa_c^+ x c^* - \kappa_c^- c - r_c c - \xi c \quad (6)$$
$$\dot{y} = r_0 c^* + r_c c - \delta y \quad (7)$$

with a mass conservation of the total amount of DNA complex constant, $c + c^* = c^{tot}$.

Numerical Simulations. Below we list the parameters used in the model for simulations.

Parameter	Value	Annotation	Other studies
ϕ, δ (1/s)	2.7×10^{-4}	degradation	$10^{-4} - 10^{-3}$ [4]
θ (M/s)	2.7×10^{-10}	transcription	$2.8 \times 10^{-11} - 2.8 \times 10^{-8}$ [5,6]
α (M/s)	5.4×10^{-10}	transcription	$2.8 \times 10^{-11} - 2.8 \times 10^{-8}$ [5,6]
k_p^+, k_c^+ (/M/s)	2.7×10^{4}	binding	$10^{4} - 10^{6}$ [7,8]
k_p^-, k_c^- (1/s)	2.7×10^{-4}	unbinding	
r_p, r_c (1/s)	2.7×10^{-3}	complex transcription	$0.05 - 0.2$ [9]*
r_0 (1/s)	2.7×10^{-4}	leakiness	
c^{tot} (nM)	1000	total DNA	
ξ (1/s)	0	abortion rate	

* Estimated from the average pause-free velocity of RNAP at saturating concentrations of nucleotide triphosphates (NTP) for an RNA length of 200 nt.

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