Supporting Information

Characterization and engineering of a Clostridium glycine riboswitch and its use to control a novel metabolic pathway for 5-aminolevulinic acid production in *Escherichia coli*

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Methods

Chemically defined M9 minimal medium (CDMM)

For the cell growth and reporter assay, bacteria were cultivated in a chemically defined M9 minimal medium (CDMM). The recipe was described as below: sterile M9 salts (1X), glucose (0.4%, w/v), MEM Amino Acids solution (Sigma M5550), Non-essential Amino Acid Solution (homemade following Sigma M7145 to eliminate glycine), RPMI-1640 Vitamins solution (Sigma R7256).

Plasmid construction

To generate pApt2-tetA-mRFP, a 286bp fragment containing synthetic promoter

BBa_J23100 and aptamer-2 (Apt2) was amplified using primers OFF-In1-F/OFF-In1-R and plasmid pGRS-Apt2-mRFP as a template; a 1294bp fragment of tetracycline efflux protein (*tetA*) was amplified using primers OFF-In2-F/ OFF-In2-R and plasmid pLacthiMtetA as a template; a 745bp *mRFP* gene was amplified using plasmid pGRS-WT-mRFP as a template with primers OFF-In3-F/OFF-In3-R; a ~2.3kb fragment of the ampicillin resistance gene (Amp^R) and pMB1 replicon was amplified using pLacthiMtetA as a template and primers OFF-Back-F/OFF-Back-R. These four fragments were purified and assembled via Gibson Assembly Master Mix of New England Biolabs (Beijing, China). After sequencing, the positive construct was designated as pApt2-tetA-mRFP.

Plasmid pApt2#82-tetA-mRFP was screened from a glycine-OFF riboswitch library constructed with primers Lib-F/Lib-R and confirmed by sequencing.

To generate pApt2#82M-tetA-mRFP, a point mutation was introduced into the aptamer element using pApt2#82-tetA-mRFP as the template and primers 82M-F/82M-R.

To generate pApt2#82-lacZ, the plasmid pApt2#82-tetA-mRFP was used as a template to amplify a ~2.4kb fragment with primers Apt-Z-VF/Apt-VR. A ~3.1kb fragment of *lacZ* gene was amplified using the pGRS-WT-lacZ plasmid as the template and primers Apt-Z-IF/Apt-Z-IR. These two fragments were purified and assembled via Gibson Assembly Master Mix. Similar approach was used for constructing pApt2#82-gfpuv. A ~2.4kb fragment was amplified using the primers Apt-gfp-VF/Apt-VR and plasmid pApt2#82-tetA-mRFP as a template; a 743bp *gfpuv* gene was amplified using the plasmid pBAD-GFPuv as a template with primers Apt-gfp-IF/Apt-gfp-IR. After purification, these two fragments were assembled using Gibson Assembly Master Mix Kit. Finally, the two new constructions were ready for use after confirmation by sequencing.

Plasmid sequence

pGRS-WT-mRFP

gctggctggattagtcctagttccgctgagggattaagttattcatttaaaagtgcatatgcattagcaaatgcaatacaaaag
cggcctgatagtattattgctgaatattatcgaaataccaataaactacggattaatataagattgaaacatctcaagtcaccatt
tatgtacaacacttttttaaggaagatagcaatgaaatcaggtttaatgagtatggatattattaattaatcattgcaaatttactag
atatgtattaatatatataacggtaaatttcaatattcagatgaagatagcgggagagttatggttatatccattcaccgaagaa
gtaaatctttcaggtatctatttaattagagatgactgctattagatgaaaccttggagagactcttgatgagcaccgaaggag
aaagtcgtacggcaaaactctcaggtaaaaggacagggaaaaggaaaagaaaaaggcagcatatttcttatcatttcttata
aaagtacttacttaaatcaattttactgtacgtctagtattacttcaatcataaaaaggtgacattgacatgaatttatcagtagtaa
tattagaaaggcggaatacatatgtcggaaaatgaaaatctatccagaatggcttcctccgaagacgttatcaaagagttcat
gcgtttcaaagttcgtatggaaggttccgttaacggtcacgagttcgaaatcgaaggtgaaggtgaaggtcgtccgtacgaa
ggcacccagaccgctaaactgaaagttaccaaaggtggtccgctgccgttcgcttgggacatcctgtccccgcagttccag
tacggttccaaagcgtacgttaaacacccggctgacatcccggactacctgaaactgtccttcccggaaggtttcaaatggg
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acgtatgtacccggaagacggtgctctgaaaggtgaaatcaaaatgcgtctgaaactgaaagacggtggtcactacgacg
ctgaagttaaaaccacctacatggctaaaaaaccggttcagctgccgggtgcttacaaaaccgacatcaaactggacatca
cctcccacaacgaagactacaccatcgttgaacagtacgaacgtgctgaaggtcgtcactccaccggtgcttaaGAAT
TCAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCC

Purple: sequence of the C. *pasteurianum* CPA27280 gene
Blue: C. *pasteurianum* glycine riboswitch element, including the promoter, riboswitch and the first nine amino acids of CPA27270
Underlined: aptamer-1 of glycine riboswitch
Wave-underlined: the short linker of the two aptamers
Double-underlined: aptamer-2 of glycine riboswitch
Red: mRFP sequence
Black, backbone sequence of plasmid

pGRS-WT-lacZ

Identical to the sequence for pGRS-WT-mRFP (above), except the mRFP sequence (red) is replaced with the β-galactosidase sequence shown below:

gccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacccaacttaatcgccttgcagcacatccccctttcgccag
ctggcgtaatagcgaagaggcccgcaccgatcgcccttcccaacagttgcgcagcctgaatggcgaatggcgctttgcct
ggtttccggcaccagaagcggtgccggaaagctggctggagtgcgatcttcctgaggccgatactgtcgtcgtcccctcaa
actggcagatgcacggttacgatgcgcccatctacaccaacgtgacctatcccattacggtcaatccgccgtttgttcccac
ggagaatccgacgggttgttactcgctcacatttaatgttgatgaaagctggctacaggaaggccagacgcgaattatttttg
atggcgttaactcggcgtttcatctgtggtgcaacgggcgctgggtcggttacggccaggacagtcgtttgccgtctgaattt
gacctgagcgcatttttacgcgccggagaaaaccgcctcgcggtgatggtgctgcgctggagtgacggcagttatctgga
agatcaggatatgtggcggatgagcggcattttccgtgacgtctcgttgctgcataaaccgactacacaaatcagcgatttcc
atgttgccactcgctttaatgatgatttcagccgcgctgtactggaggctgaagttcagatgtgcggcgagttgcgtgactac
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cccgtacgtcttcccgagcgaaaacggtctgcgctgcgggacgcgcgaattgaattatggcccacaccagtggcgcggc
gacttccagttcaacatcagccgctacagtcaacagcaactgatggaaaccagccatcgccatctgctgcacgcggaaga
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ccagctgagcgccggtcgctaccattaccagttggtctggtgtcaaaaataa

pApt2-tetA-mRFP

cacatttccccgaaaagtgccacctgacgtctaagaaaccattattatcatgacattaacctataaaaataggcgtatcacga
ggccctttcgtcttcacctcgagagcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggctttacactttat
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tacatcgttatggcaggagcaaactatgcaagtcgacctgctggatccaaaatctaacaatgcgctcatcgtcatcctcggc
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gcatcgccagtcactatggcgtgctgctagcgctatatgcgttgatgcaatttctatgcgcacccgttctcggagcactgtcc
gaccgctttggccgccgcccagtcctgctcgcttcgctacttggagccactatcgactacgcgatcatggcgaccacaccc
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actgaaagacggtggtcactacgacgctgaagttaaaaccacctacatggctaaaaaaccggttcagctgccgggtgctta
caaaaccgacatcaaactggacatcacctcccacaacgaagactacaccatcgttgaacagtacgaacgtgctgaaggtc
gtcactccaccggtgcttaatctagaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttg
tttgtcggtgaacgctctcctgagtaggacaaatccgccgccctaga
Underlined: synthetic promoter of BBa-J23100
Double-underlined: aptamer-2 (Apt2) of glycine riboswitch
Green: sequences of the expression platform of glycine riboswitch
Blue: sequence of *tetA* gene
Red: mRFP sequence
Black, backbone sequence of plasmid

pApt2#82-tetA-mRFP
Identical to the sequence for pApt2-tetA-mRFP (above), except the sequence of the expression platform (green) is replaced shown below (Table S3):
AAAACCCTTCTCGAACT

pApt2#82-gfpUV
cacatttccccgaaaagtgccacctgacgtctaagaaaccattattatcatgacattaacctataaaaataggcgtatcacga
ggccctttcgtcttcacctcgagagcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggctttacactttat
gcttccggctcgtatgttgtgtggaattgtgagcggataacaattgaattcaacttgacggctagctcagtcctaggtacagtg
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acgcactatatctttcaaagatgacgggaactacaagacgcgtgctgaagtcaagtttgaaggtgataccttgttaatcgtat
cgagttaaaaggtattgattttaaagaagatggaaacattctcggacacaaactcgagtacaactataactcacacaatgtata
catcacggcagacaaacaaaagaatggaatcaaagctaacttcaaaattcgccacaacattgaagatggatccgttcaact
agcagaccattatcaacaaaatactccaattggcgatggccctgtccttttaccagacaaccattacctgtcgacacaatctg
ccctttcgaaagatcccaacgaaaagcgtgaccacatggtccttcttgagtttgtaactgctgctgggattacacatggcatg

gatgagctctacaaataatctagaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgttt
gtcggtgaacgctctcctgagtaggacaaatccgccgccct
Underlined: synthetic promoter of BBa-J23100
Double-underlined: Apt2#82
Green: sequences of *gfpUV* gene
Black, backbone sequence of plasmid

pApt2#82-lacZ
Identical to the sequence for pApt2#82-gfpUV (above), except the *gfpUV* sequence (green) is replaced with the β-galactosidase sequence of pGRS-WT-lacZ.

Table S1. Strains and plasmids used in this study

Strains or plasmids	Major characteristics[a]	Source or reference
Strains		
E. coli		
W3110	*F⁻, λ⁻, rph-1*	Lab collection
TOP10	F⁻*mcrAΔ(mrr-hsdRMS-mcrBCΦ80lacZΔM15Δ lacX74 recA1 ara D139(araleu)7697galU galK rpsL(StrR) endA1 nupG*	Lab collection
W3-DZ	W3110 Δ*lacZ*, Kan^R	This study
BL21 (DE3)	F^- *omp*T *hsd*S_B (r_B^-, m_B^-) *gal dcm* (DE3)	Lab collection
BL21-hemA	BL21(DE3) harboring pETduet-*hemA*	[1]
BL21-E3	BL21(DE3) harboring pETduet-*hemA* and pRSFduet-*aceA-agxt*	[1]
BL21-Apt2#82	Apt2#82-*hemB*, derived from BL21 (DE3)	This study
BL21-Apt2#82-hemA	BL21-Apt2#82 harboring pETduet-1-*hemA*	This study
BL21-Apt2#82-E3	BL21-Apt2#82 harboring pETduet-1-*hemA* and pRSFduet-*aceA-agxt*	This study
Plasmids		
pGRS-WT-lacZ	Wild-type C. *pasteurianum* glycine riboswitch fused with *lacZ*	This study
pGRS-WT-mRFP	Wild-type C. *pasteurianum* glycine riboswitch fused with *mRFP*	This study
pGRS-Apt1-mRFP	Aptamer-1 of C. *pasteurianum* glycine riboswitch fused with *mRFP*	This study
pGRS-Apt2-mRFP	Aptamer-2 of C. *pasteurianum* glycine riboswitch fused with *mRFP*	This study
pGRS-Del	Wild-type C. *pasteurianum* glycine riboswitch, but deleted two aptamers, fused with *mRFP*	This study
pGRS-tetA-mRFP	Wild-type C. *pasteurianum* glycine riboswitch fused with *tetA-mRFP*	This study
pApt2-tetA-mRFP	Aptamer-2 of C. *pasteurianum* glycine riboswitch fused with *tetA-mRFP*	This study
pApt2#82-tetA-mRFP	Synthetic glycine-OFF riboswitch Apt2#82 fused with *tetA-mRFP*	This study
pApt2#82M-tetA-mRFP	A point mutation in pApt2#82-tetA-mRFP	This study
pApt2#82-gfpuv	Synthetic glycine-OFF riboswitch Apt2#82 fused with *gfpuv*	This study

pApt2#82-lacZ	Synthetic glycine-OFF riboswitch Apt2#82 fused with *lacZ*	This study
pETduet-*hemA*	pETduet-1 containing hemA	[1]
pRSFduet-*aceA-agxt*	pRSFduet-1 containing aceA and agxt	[1]
pRedCas9	λ Red expression cassette combined CRISPR system, Spc^R	[2]
pGRB	synthetic guide RNA plasmid	[2]
pGRB-hemB	sgRNA for hemB *gene*	[2]

[a]Abbreviations: Kan^R, Kanamycin resistance; Spc^R, spectinomycin resistance

Table S2. Primers used in this study.

Name	Sequence (5'-3')
CpRS-lacZ-F	gctggctggattagtcctag
CpRS-lacZ-R	ctctggatagattttcattttccg
lacZ-CpRS-F	cggaaaatgaaaatctatccagagccgtcgttttacaacgtc
lacZ-CpRS-R	ctaggactaatccagccagcggcacttttcggggaaatg
RFP-CPRS-F	atgtcggaaaatgaaaatctatccagaatggcttcctccgaagac
RFP-CPRS-R	agctgtgaccgtctgaattc
CPRS-RFP-F	gaattcagacggtcacagcttgtc
CPRS-RFP-R	tctggatagattttcattttccgacat
del-RS-F	gaaaaggaaaagaaaaaggcagc
del-RS-R	cttcatctgaatattgaaatttaccg
del-RS1-F	gctattagatgaaaccttggagag
del-RS2-R	gtttcatctaatagcagtcatctc
Lib-F	NNNNNNNNNNNNNNgcaggagcaaactatgcaag
Lib-R	NNNNNNNNNNNNNNccttttccctgtccttttacc
OFF-In1-F	ttgacggctagctcagtcctaggtacagtgctagcaatattcagatgaagtattagatgaaacc
OFF-In1-R	Atgtattccgcctttctaatattactactg
OFF-In2-F	cagtagtaatattagaaaggcggaatacatcgttatggcaggagcaaactatgcaag
OFF-In2-R	cctcctccagatcctcctccagatccacctccaagggtcgaggtggcccggc
OFF-In3-F	gaggaggatctggaggaggttctggaggaggttctaagcttatggcttcctccgaagacg
OFF-In3-R	ctttcgttttatttgatgcctctagattaagcaccggtggagtgacg
OFF-Back-F	Taatctagaggcatcaaataaaacgaaag
OFF-Back-R	gactgagctagccgtcaagttgaattcaattgttatccgctc
82M-F	Gaaaccttgtagagactcttgatgagcacc
82M-R	Gtctctacaaggtttcatctaatacttcatc
Apt-Z-VF	cagttggtctggtgtcaaaaataatctagaggcatcaaataaaacgaaag
Apt-VR	Agtttgctccttagttcgagaag
Apt-Z-IF	cttctcgaactaaggagcaaactgtcgttttacaacgtcgtgac
Apt-Z-IR	Ttatttttgacaccagaccaactg
Apt-gfp-VF	gcatggatgagctctacaaataatctagaggcatcaaataaaacgaaag
Apt-gfp-IF	cttctcgaactaaggagcaaactgtcgttttacaacgtcgtgac
Apt-gfp-IR	Ttatttgtagagctcatccatgc
hemB-1F	gtgatagccagagtgcaagc
hemB-1R	gtatctttaaagcccgcagc
OE-DN-F	cggcaaaactctcaggtaaaaggacagggaaaacccttctcgaactaaggagagcttatgacagacttaatccaacgcc
OE-Up-R	cttttacctgagagttttgccgtacgactttctccttcggtgctcatcaagagtctctccaaggtctgcctgatgtttgtgg
sg-hemB-F1	gtcctaggtataatactagtcagaccatgacagacttaatcgttttagagctagaaatagcaag

sgRNA-R	actagtattatacctaggactgagc

Table S3. Sequences of selected glycine-OFF riboswitches

Glycine-OFF clone #	Sequence		
Library*	**AGGG**	NNNNNNNNNNNNNNNNNN	***A**AGGAG**A***
2		GGAAGGAACCCAGC	
5		GCTAAGAGCATAG	
17		GTTCGTTACTCGGC	
22		GGCCCGGGAAACCTGC	
26		ATGATTTCGAAAGTGT TC	
31		GCCTGCTCAGACTTGTAGT	
53		TCCTTGGATCAGACCGGC	
82		AAAACCCTTCTCGAACT	
108		TAGTCTCGGCCACT	
116		GAGCGCAATCTA	
137		TCAGCAACCCGTGC	
165		CACGCAGGGGCAT	
182		ACGTCCCGGGATCCT	
236		CCGTAATCCTA	
258		CAAATGGTCCCACG	
272		GTATACGA	
317		ATAGACAGACTTACTC	
356		GACCGATCCATTCGATG	

*The flanking constant sequences are shown in bold. The putative SD sequence is italicized.

Figure S1. (A) Nucleotide sequence alignment of putative glycine riboswitches from *C. pasteurianum*, *C. acetobutylicum, V. cholerae, S. pyogenes, Cand.* P. ubique and *B. subtilis*. Putative base pairing in individual aligned sequences were depicted with appropriate colors. (B) A neighbor-joining phylogenetic tree inferred from aligned glycine riboswitches sequences. (C) Schematic representation of the glycine riboswitch locus in *C. pasteurianum*. Apt-1 and Apt-2 represent two aptamers of glycine riboswitch.

(A) glycine riboswitch alignment

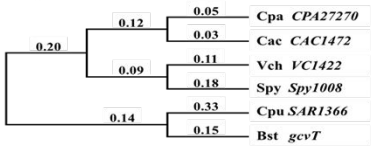
```
Consensus    ----------WRSR*****----------------------------------------*R**
Cpa CPA27270 AUGAAGAUAGCGGGAGAGUUAUGGU--------------------UAUAUCCAU--UCACC
Cac CAC1472  AGAUGAAGUUAGCGGGAGAGCUUUGGC--------------------UUUUGCCAUACACC
Vch VC1422   GUUGAAGACUGCAGGAGAGUGGUUGUUAACCAGAUUUUAACAUCUGAGCCAAAUAACCCGCC
Spy Spy1008  AAUGAUGUCAUGCAGGAGA-------A---GAA---UUU---------------UUUUCGCC
Cpu SAR1366  --UUCUUAUAUACGGGAGAGA--CUAC-A----------AACAAUC--------GUAGCGCC
Bst gcvT     ---AUGACAGCAAGGGGAGAGA-CCUG--A--CCGA---AAACC--UCGGGAUACAGGCGCC

Consensus    ****R**YW*--------------**Y*****Y-----------------------------
Cpa CPA27270 GAAGAAGUAA------------AUCUUUCAGGUAUC-UA---UUUA----------------
Cac CAC1472  GAAGAAGUAA------------AUCUUUCAGGUAUC-UA---UUUA----------------
Vch VC1422   GAAGAAGUAA------------AUCUUUCAGGU-GCAUUAUUCUUAGCCAUAUAUUGGCAAC
Spy Spy1008  GAAGGAGUUA-----------UA-CUCUCAGGUGUUUCAGUUU--UUG---------------
Cpu SAR1366  GAAGGAGCAA--CCACCCAGGAAUCUCUCAGGC-----------------------------
Bst gcvT     GAAGGAGCAAACUGCGGAGUGAAUCUCUCAGGC-----------------------------

Consensus    ------RM-RK-**YSYW--WYR----R*-YYY****R*-------------W--------*
Cpa CPA27270 --AUUAGAGAUGACUGCU-AUUAGAUGAAACCUUGGAGAGACUCU-----UGA----UGAGC
Cac CAC1472  --AUUAGAGAUGACCGCU-AUUGGAUGAACCCUUGGAGAGACUCU-----UAA----AGAGC
Vch VC1422   GAAUAAGCGAGGACUGUA-GUUGGAGGAACCUCUGGAGAGAACCGUU---UAAU---CGGUC
Spy Spy1008  -----AAC-GGGACUGUUUGAUGGACGGACUUCUGGAGAGACCUU-----AUU-----AGGC
Cpu SAR1366  ------AAAAGGACCGUAACAUA-UU-AA-CUCUGGAAA--GAGAU----UAA---GUUCUC
Bst gcvT     ------AAAAGAACUCUUGCUCGACGCAA-CUCUGGAGAGUGUUUGUGCGGAUGCGCAAACC

Consensus    -**R*M**R--M----------------------**W**Y*****YRMMMRK****R----
Cpa CPA27270 ACCGAAGGAGAAAGUCGU--------ACGGCAAAAAACUCUCAGGUAAAAGGACAGGGAAAA
Cac CAC1472  ACCGAAGGAGAAAGCAUAAAAAAA-------GCGAAACUCUCAGGUAAAAGGACAGGGGACA
Vch VC1422   GCCGAAGGAGCAAGCUCUGCGCAUAUGCAGAGUGAAACUCUCAGGCAAAAGGACAGAGGAGU
Spy Spy1008  -CCGAAGGG-GCAAGGCA---UAC---UGC--UCAAUCUCUCAGGCAAAAGGACAGAAGGUA
Cpu SAR1366  GCCGACGGAAUA----------------------AAACUCUCAGGCAAACAUACAGAUGGGU
Bst gcvT     ACCAAAGGGG-ACGUCUUUGC--GUAUGCAAAGUAAACUUUCAGGUGCCAGGACAGAGAACC
```

(B) phylogenetic tree



(C) chromosome

Figure S2. Primary DNA sequence of the 5'UTR regulatory region and its neighboring region of the Apt2-tetA-mRFP library.

BBa_J23100 promoter

tgaattcaacttgacggctagctcagtcctaggtacagtgctagcaatattcagatgaagtattagatgaa

Apt2 aptamer

a**ccttggagagactcttgatgagcaccgaaggagaaagtcgtacggcaaaactctcaggtaaaa**

Randomized region

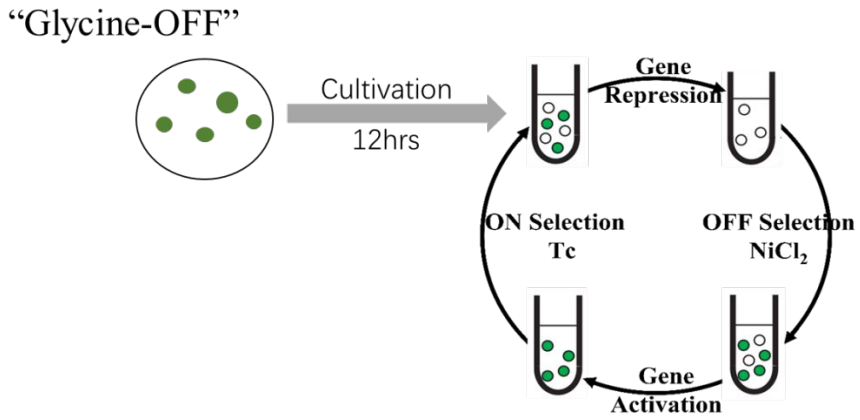
ggacagggaaaaggNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNgc

SD sequence

aggagcaaactatgcaagtcgac

Start codon

Figure S3. Schematic illustration of dual genetic selection scheme to identify glycine-OFF riboswitches. A library of candidate riboswitches was constructed under selective conditions in the presence of 0.1 mM glycine to repress *tetA* gene. Following *tetA* repression, the clones were then cultured in the presence of 0.1 mM glycine and 0.3 mM $NiCl_2$ to select glycine-OFF riboswitches. Surviving clones were cultivated in the absence of glycine to allow *tetA* expression to readjust. Clones were then grown on media containing 30 μg/mL tetracycline but without glycine. Only clones displaying higher levels of *tetA* expression could survive the selection step.



Reference

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(2) Li, Y., Lin, Z., Huang, C., Zhang, Y., Wang, Z., Tang, Y. J., Chen, T., and Zhao, X. (2015) Metabolic engineering of *Escherichia coli* using CRISPR-Cas9 meditated genome editing. *Metab. Eng. 31*, 13-21.