

Supporting Information

Development of a genetically-encoded biosensor for reporting the methyltransferase-dependent biosynthesis of semi-synthetic macrolide antibiotics

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Supplementary Table S1. Sequences of DNA constructs. RBS for each gene of interest is underlined. The promoter region for each gene of interest is bold italicized. Mutations are bolded and underlined.

Construct Name	DNA Sequence
pMLGFP	<pre> GGTAATACGGTTATCCACAGAATCAGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGA ACCGTAAAAGGCCGCGTTGCTGGCGTTTCATAGGCTCCGCCCTGACGAGCATCACAAAATGACGCTCAAG TCAGAGGTGGCGAAACCGACAGGACTATAAGATAACCAGGCCTTCCCTCGGAAGCGTGGCGCTTCATAGCTCACGCTGTAG CCGACCCCTGCCGCTTACCGATACTGTCCGCCCTCCCTCGGAAGCGTGGCGCTTCATAGCTCACGCTGTAG GTATCTCAGTCGGTGTAGGTCGTTCGCTCAAGCTGGCTGTGACGAACCCCCCGTTAGCCGACCGCTGCC TTATCCGTAACATCGTCTTGAGTCCAACCCGTAAGACACGACTTATGCCACTGGCAGCAGCCACTGGTAACAGGA TTAGCAGAGCGAGGTATGAGGCGGTGCTACAGAGTTCTGAAGTGGTGGCTAACTACGGCTACACTAGAAGGACAG TATTGGTATCTCGCTCTGCTGAAGCCAGTTACCTCGGAAAAAGAGTTGGTAGCTTGTGATCCGAAACAAACAC CGCTGGTAGCGGTGGTTTTTGTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTAAGAAGATCCTTGATC TTTCTACGGGTCTGACGCTCAGTGGAACGAAACTCACGTTAAGGGATTTGGTCATGAGATTATCAAAAGGATCT TCACCTAGATCCTTAAATTAAAAATGAAGTTAAATCAATCTAAAGTATATGAGTAAACTGGTCTGACAGTTACC AATGCTTAATCAGTGAGGCACCTATCAGCGATCTGCTATTCGTTCATCCATAGTGCCTGACTCCCCGCTGTAGA TAACTACGATAACGGAGGGCTTACCATCTGCCAGTGCTGCAATGATAACCGCGAGACCCACGCTACCGCTCCAGA TTTATCAGCAATAACCAAGCCAGCCGGAAAGGGCGAGCGCAGAAGTGGCCTGCAACTTATCCGCTCCATCCAGTCT ATTAATTGTTGCCGGAAAGCTAGAGTAAGTAGTTGCCAGTTAATAGTTGCGCAACGTTGGCATTGCTACAGGCA TCGTGGTGTACGCTCGTGTGGTATGGCTTATTAGCTCCCTCGTCCTCGATCGTTGTCAGAAGTAAGTGGCCGAGTGT ATGTTGTGCAAAAAGCGGTTAGCTCCTCGTCCTCGATCGTTGTCAGAAGTAAGTGGCCGAGTGTATCACTCAT GGTTATGGCAGCACTGCATAATTCTTACTGTCATGCCATCCGTAAGATGCTTCTGTGACTGGTAGACTCAACCA AGTCATTCTGAGAATAGTGTATGCCGACCGAGTTGCTCTGCCGGCGTCAACACGGATAATACCGGCCACATAG CAGAACTTAAAGTGCATCATTGGAAAACGTTCTCGGGCGAAAACGTTCAAGGATCTACCGCTGGTAGATCC AGTCGATGTAACCACTCGTCACCCACTGATCTTCAGCATCTTACTTCAACCGCTTCTGGGTAGCAAAAC AGGAAGGCAAAATGCCAAAAAGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTCCTTTCAATAT TATTGAAGCATTATCAGGGTTATTGTCATGAGCGGATACATATTGAATGTATTAGAAAATAACAAAAGAGTT TGTAGAAACGCAAAAGGCCATCCGTCAAGGATGGCTTCTGCTTAATTGATGCCGGCAGTTATGGCGGGCGTCTG CCGCCACCCCTCCGGGCCCTGCTCGCAACGTTCAAATCCGCTCCGGCGATTGCTACTCAGGAGAGCGTTCACC GACAAACAACAGATAAAACGAAAGGCCAGTCTCGACTGAGCCTTCGTTATTGATGCCGGCAGTTCCCTACTC TCGCATGGGAGACCCACACTACCATCGCGCTACGGCGTTCACTCTGAGTCGGCATGGGTAGGTGGACCA CCGCGCTACTGCCGCCAGGCAAATTGTTATCAGACCGCTTCTGCGTTGATTAATCTGTATCAGGCTGAAAATCT TCTCTCATCCGCCAAAACAGCCAAGCTGGAGACCGTTAAACTCAATGATGATGATGATGGTCACGGCGCTATT AGATCCTCTGAGATGAGTTTGTGCGGGCCAAGCTTATTGATGAGCTCATCCATGCCATGTGAAATCCCAGCAG CAGTTACAAACTCAAGAAGGACCATGTGGTCACGCTTCTGGGATCTTCGAAAGGGCAGATTGTCGACAGGTA </pre>

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pSense-3	ATAACGCAGGAAAGAACATGTGAGCAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAGGCCGTTGCTGGCGTT TTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTAT AAAGATACCAGGCAGTCCCTGGAGCTCCCTGTGCGCTCTCTGTTCCGACCCCTGCCGTTACCGGATACCTGTCC GCCTTCTCCCTCGGGAAAGCGTGGCGTTCTCATAGCTCACGCTGTAGGTATCTCAGTCGGTAGGTCGTTGCTC CAAGCTGGCTGTGACGAACCCCCCGTTCAGCCGACCGCTGCCCTATCCGTAACATACGTCTTGAGTCCAACC CGGTAAGACACGACTTATGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTA CAGAGTTCTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTGGTATCTGCCTCTGCTGAAGCCAGT TACCTCGAAAAAGAGTTGGTAGCTCTGATCCGAAACAAACCACCGCTGGTAGCGTGGTTTTGTTGCAAG CAGCAGATTACGCGCAGAAAAAAAGGATCTAAGAAGATCCTTGATCTTCTACGGGCTGACGCTCAGTGGAACG AAAACACGTTAAGGGATTGGTATGAGATTATCAAAAAGGATCTCACCTAGATCCTTAAATTAAAAATGAAGT TTAAATCAATCTAAAGTATCCATGGATATGAGTAAACTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTAT CTCAGCGATCTGCTATTCGTTCATCCATAGTGCCTGACTCCCCGTCGTAGATAACTACGATAACGGGAGGGCTTAC CATCTGGCCCCAGTGCCTGCAATGATAACCGCAGACCCACGCTCACCGGCTCAGATTATCAGCAATAAACAGCCAGC CGGAAGGGCCAGCGCAGAAGTGGCCTGCAACTTATCCGCTCCATCCAGTCTATTAAATTGTTGCCGGGAAGCTAGA GTAAGTAGTTGCCAGTTAATAGTTGCGAACGTTGCTACAGGCATCGTGGTAGCAGCTCGTGTGTTGG TATGGCTTCATTAGCTCCGTTCCAAACGATCAAGGCAGTTACATGATCCCCATGTTGCAAAAAGCGGTTAGCT CCTTCGGCTCCGATCGTGTAGAAGTAAGTTGGCCAGTGTACTCATGGTTATGGCAGCACTGCATAATTCT CTTACTGTCATGCCATCCGTAAGATGCTTCTGTGACTGGTAGACTCAACCAAGTCATTGAGAATAGTGTATGCG GCGACCGAGTTGCTTGGCCGGCTCAACACGGGATAATACCGGCCACATAGCAGAACCTTAAAGTGTCTCATCATT GGAAAACGTTCTCGGGCGAAAACCTCAAGGATCTTACCGCTGTTGAGATCCAGTGTGAAACCCACTCGTCAC CCAACGTCTCAGCATCTTACTTCACCAGCGTTCTGGTGAGCAAAACAGGAAGGCAAAATGCCAAAAAA GGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTCCTTTCAATATTGAAGCATTACAGGGTTATT GTCTCATGAGCGGATACATATTGAATGTATTAGAAAATAAACAAAAGAGCATGCGTTGTAGAAACGCAAAAG GCCATCCGTCAGGATGGCCTCTGCTTAATTGATGCCTGGCAGTTATGGCGGGCTCTGCCGCCACCCCTCCGGGC CGTTGCTTCGCAACGTTCAAATCGCTCCGGGAGTTGCTACTCAGGAGAGCGTTACCGACAAACAACAGATAA AACGAAAGGCCAGTCTCGACTGAGCCTTCGTTATTGATGCCTGGCAGTCCCTACTCTGCATGGGAGACCC CACACTACCACGGCGCTACGGCGTTCACTCTGAGTCGGCATGGGTCAGGTGGGACCACCGCGCTACTGCCGCCA GGCAAATTCTGTTTATCAGACCGCTCTGCGTTGATTTAATCTGTATCAGGCTGAAAATCTCTCATCCGCCAAA CAGCCAAGCTGGAGACCGTTAAACGGGCCAAGCTTTGAGGCTCATCCATGCCATGTGTAATCCAGCAGCAGT TACAAACTCAAGAAGGACCATGTGGTCACGCTTCGTTGAGTCGGCATGGGTCAGGTGGGACCACCGCGCTACTGCCGCCA TTGTCGGTAAAGGACAGGGCCATGCCAATTGGAGTATTGTTGATAATGGTCTGCTAGTTGAACGGAACCACATT CAACGTTGTCGGGAATTGAGTTAGCTTGTGATTCCATTCTTGTGCTGCCGTGATGTACATTGTGAGTTAA AGTTGACTCGAGTTGTGTCAGAATGTTCCATCTCTTAAATCAATACCCCTTAACCGATACTCGATCGATTAACAAGGG TATCACCTCAAACCTGACTTCAGCACCGCTTGTAGGTCCGTCATCTTGAAAGATATAGTGCCTCTGTACATAAC

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Supplementary Table S2. Strains and plasmids used in this study.

Bacterial Strain/plasmid	Genotype/description	Reference
<i>E. coli</i> ® 10G	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) <i>endA1 recA1</i> φ80d/ <i>lacZΔM15</i> Δ/ <i>lacX74</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL(Str^R) nupG λ-tonA</i>	Lucigen
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80/ <i>lacZΔM15</i> Δ/ <i>lacX74</i> <i>recA1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK λ- rpsL(Str^R) endA1 nupG</i>	Invitrogen
<i>E. coli</i> BL21 (DE3)	Used to express MphR for structure determination	Promega
pMLGFP	Houses the reporter module ($P_{MphR}-gfp$) and <i>MphR</i> . Amp resistance.	Gardner et al., Mol Biosyst. 2011, 7, 2554
pMLGFPK	Derivative of pMLGFP with inserted <i>KpnI</i> restriction site to facilitate cloning mutant MphR genes. Amp resistance.	This study
pJZ12	Carries the macrolide phosphotransferase MphA. Tet resistance.	Gardner et al., Mol Biosyst. 2011, 7, 2554
pCOLADuet-EryG	Derivative of pCOLADuet containing wild-type EryG	This study
pSense-MphR-MphA	Derivative of pSense containing wild-type MphR and MphA	This study
pSense-MphA-MphR-RBScalculate	Derivative of pSense-MphA-MphR with a calculated RBS	This study
pSense-1	Derivative of pSense-MphA-MphR-RBScalculate containing EryG	This study
pSense-2	Derivative of pSense-1 containing the pET28a RBS (AAGGAG) for EryG	This study
pSense-3	Derivative of pSense-2 containing the T7 promoter driving EryG	This study
pSense-M9C4-E7-MphA-EryG	Derivative of pSense-3 containing M9C4 MphR with the E7 RBS upstream to it	This study

Supplementary Table S3. Oligonucleotides used in this study.

Entry	Primer name	Sequence
1	MphR_SSM_R122_F	TACGCACGCTTGCATCCAGNNKAACCGCGCGGTGGAGGGGAT
2	MphR_SSM_R122_R	ATCCCCTCCACCACCGCGCGGTTMNNCTGGATCGCAAGCGTGCATA
3	MphR_SSM_N123_F	ACGCTTGCATCCAGCGNNKCGCGCGGTGGAGGGGAT
4	MphR_SSM_N123_R	ATCCCCTCCACCACCGCGCGMNNCCGCTGGATCGCAAGCGT
5	MphR_SDM_R122I_F	TACGCACGCTTGCATCCAGATTAAACCGCGCGGTGGAGGG
6	MphR_SDM_R122I_R	CCCTCCACCACCGCGCGGTTAATCTGGATCGCAAGCGTGCATA
7	MphR_SDM_R122P_F	TACGCACGCTTGCATCCAGCCGAACCGCGCGGTGGAGGG
8	MphR_SDM_R122P_R	CCCTCCACCACCGCGCGGTTGGCTGGATCGCAAGCGTGCATA
9	MphR_SDM_R122N_F	TACGCACGCTTGCATCCAGAACAAACCGCGCGGTGGAGGG
10	MphR_SDM_R122N_R	CCCTCCACCACCGCGCGGTTGGCTGGATCGCAAGCGTGCATA
11	MphR_SDM_R122K_F	TACGCACGCTTGCATCCAGAAGAACCGCGCGGTGGAGGG
12	MphR_SDM_R122K_R	CCCTCCACCACCGCGCGGTTCTCTGGATCGCAAGCGTGCATA
13	MphR_SDM_K132N_F	TGGAGGGGATCCGCAATCGACTGCCCGAGGTGCTC
14	MphR_SDM_K132N_R	GAGCACCTGGGGCAGTCGATTGCGGATCCCCCTCCA
15	MphR_SDM_A151T_F	TGCTCCTGCACTCGGTATCAGTGCGCGACGATGCAGTGG
16	MphR_SDM_A151T_R	CCACTGCATCGCGCCAGTGATGACCGAGTGCAGGAGCA
17	MphR_SDM_H184Q_F	TGTTTAATGTTCCGAACAAGACGATTCCAACCTCCA
18	MphR_SDM_H184Q_R	TGGAGGAGTTGAAATCGTCTGTTGGAAACATTAAACA
19	NCR	GATAGCGCCCCCAACCCATACAGAACGGTGAACACTGATGC
20	NCR	GCATCAGTGTTCACCTCTGTATGGTTGGGGCGCTATC
21	K132N	TGGAGGGGATCCGCAATCGACTGCCCGAGGTGCTC
22	K132N	GAGCACCTGGGGCAGTCGATTGCGGATCCCCCTCCA
23	A151T	TGCTCCTGCACTCGGTATCAGTGCGCGACGATGCAGTGG
24	A151T	CCACTGCATCGCGCCAGTGATGACCGAGTGCAGGAGCA
25	H184Q	TGTTTAATGTTCCGAACAAGACGATTCCAACCTCCA
26	H184Q	TGGAGGAGTTGAAATCGTCTGTTGGAAACATTAAACA
27	R122I	TACGCACGCTTGCATCCAGATTAAACCGCGCGGTGGAGGG
28	R122I	CCCTCCACCACCGCGCGGTTAATCTGGATCGCAAGCGTGCATA
29	MphR_INS_Kpn1_F	CGTCGTCGATTAGGTACCGGTACGG
30	MphR_INS_Kpn2_R	CCGTACCGGTACCTAATCGACGACG
31	MphR_EPPCR_F	CGAGCTCTAGATGGTGCAAAACCTTC
32	MphR_EPPCR_R	CTGCGTTATCCCCTGATTCT

33	CL2056	AAAccatggGGCATCATCATCATCATCATTGGAAAACCTATACTTCAAGGCCCCGCC AAGCTCAAG
34	CL2057	TTTgaattccGGGCCGCTACTAGTTACGCATGTGCCTGGAGG
35	CL2062	ATGATGATGATGATGCCcatggtatatctccttcttaagt
36	CL2063	CTCCAGGCACATCGCGTAAGaaattcGAGCTCCGTCGACAAGCTTGCin
37	A3_RBS_mutation_1	TAGCGCCCCCAACCCATACGGAAGGTGAACACTGATGC
38	A3_RBS_mutation_2	GCATCAGTGTTCACCTCCGTATGGGTTGGGGGGCGCTA
39	E7_RBS_mutation_1	GATAGCGCCCCCAACCCATACAGATGGTGAACACTGATGC
40	E7_RBS_mutation_2	GCATCAGTGTTCACCATCTGTATGGGTTGGGGGGCGCTATC
41	H4_RBS_mutation_1	CCCAACCCATACAGAAGGCGAACACTGATGCC
42	H4_RBS_mutation_2	GGCATCAGTGTCGCCCTCTGTATGGGTTGGG
43	EryG_PCR_NcoI	ACTCACTATAGGGGAATTGTGAGCGGATAAC
44	EryG_PCR_HindIII	GCAAGCTTGTGACCGGAGCTC
45	MphR.F	TGGCATGATAGCGCCCCCAACCCATA
46	MphR.R	GGGTTTAGATTTGGATACTATGTTACGCATGTGC
47	MphA.F	CATAGTATCCAAAATCTAACCCGTCA
48	MphA.R	GGGACTCTGCACACCTCATTCCGTCGGCG
49	pSENSE.F	GGCGCTATCATGCCATAC
50	pSENSE.R	GGTGTGCAGAGTCCCTGC
51	CalculRBS_MCS	<u>GGAGATCCTCACCCGAAATTCTAaggaggaGACCATATGGAATTGGTACCTACGGTCGG</u> <u>TGGTATCGAAG</u>
52	pSense_MphAR_GA_1	ATATACATATGAGCGTGAAGCAGAAGTCAGC
53	pSense_MphAR_GA_2	ATATAGGTACCCGGATCTCAGTGGTGGTGG
54	EryG_PCR_NdeI	ATATACATATGAGCGTGAAGCAGAAGTCAGC
55	pET28a_KpnI	ATATAGGTACCCCAATCCGGATATAGTTCCCTTTCAG
56	pET28a_EryG_GA_1	CGTCGTCGATTAGTACCCCTCTAGAAATAATTTGTTAAC
57	pET28a_EryG_GA_2	GTACCGAATTCCATATGGAATTGGATCCCTACGC
58	pET28a_EryG_GA_3	CGTCGTCGATTAGTACCCGCGAAATTAAATACGAC
59	pSense_MphAR_RBScal_culated_GA_1	GCGTAGGGATCCGAATTCCATATGGAATTGGTACCTACG
60	pSense_MphAR_RBScal_culated_GA_2	AACAAAATTATTCTAGAGGGGACTAATCGACGACGGTC
61	pSense_MphAR_RBScal_culated_GA_3	GTCGTATTAATTGCGGGTACTAATCGACGACGGTC

62	pMLGFPK_E7M9C4_G	TGGCATGATAGCGCC
	A_1	
63	pMLGFPK_E7M9C4_G	GTTTAGATTTGGATACTATGTTACGCATGTGCCTGG
	A_2	
64	pSense_3_GA_1	CATAGTATCCAAAATCTAAACCGTCAAG
65	pSense_3_GA_2	GGCGCTATCATGCCATACC

Supplementary Table S4. Data collection and refinement statistics.

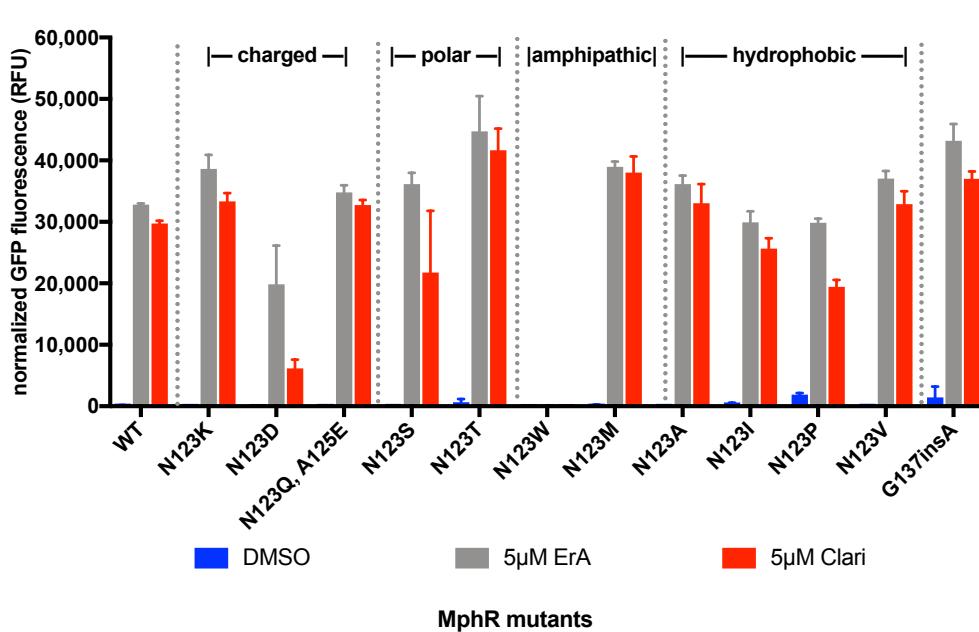
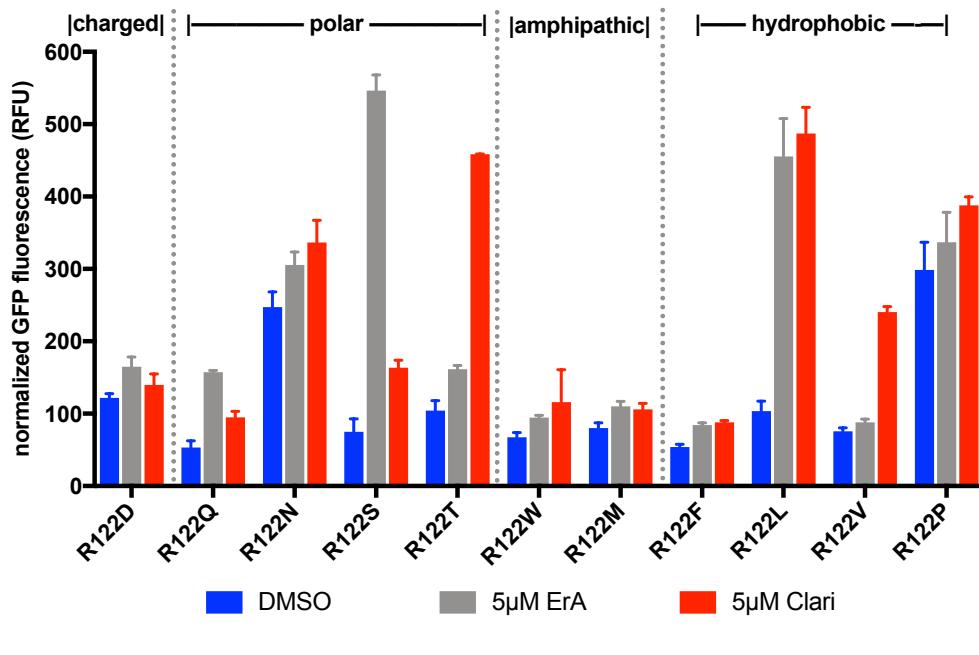
MphR(A)-CLA	
Protein Data Bank code	6U18
<i>Data collection</i>	
Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell dimensions	
a, b, c (Å)	42.90, 53.16, 158.82
α, β, γ (°)	90.0, 90.0, 90.0
Resolution (Å)	2.00 (29.14 – 2.00) ^a
Completeness (%)	99.9 (100.0) ^a
Redundancy	6.1 (5.7) ^a
R _{merge}	0.048 (0.292) ^a
<I> / <σ(I)>	(22.8) (5.3) ^a
CC _{1/2}	0.999 (0.959) ^a
<i>Refinement</i>	
Resolution (Å)	29.14 – 2.00
No. reflections	25416
No. atoms (protein)	6038
R _{work} / R _{free}	0.202 / 0.221
Wilson <B-factor> (Å)	23.9
B _{ave} (all atoms)	33.0
RMS deviations	
Bond lengths (Å)	0.28
Bond angles (°)	0.41

^a Values in parentheses are for highest resolution shell

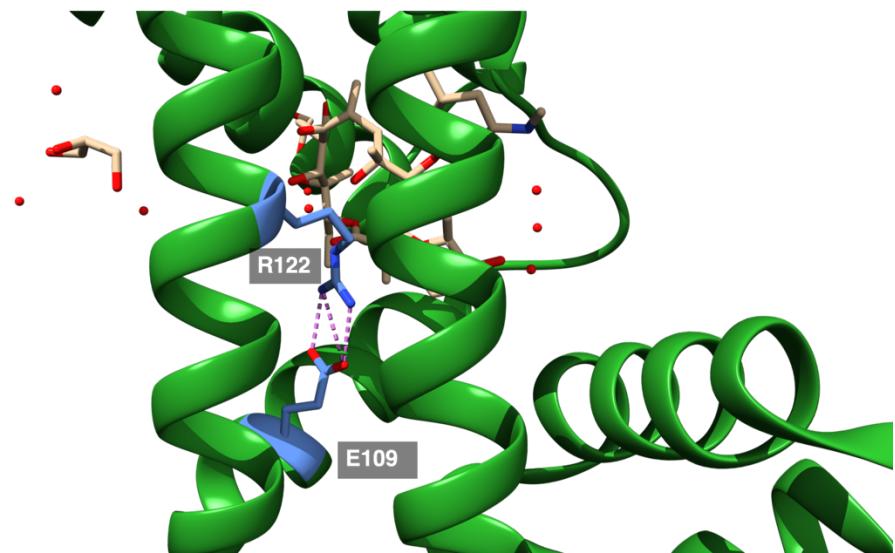
Supplementary Table S5. LC-HRMS gradient and scan parameters.

Time (min)	% B
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8.0	80.0
10.0	80.0
10.1	2.0
12.0	2.0

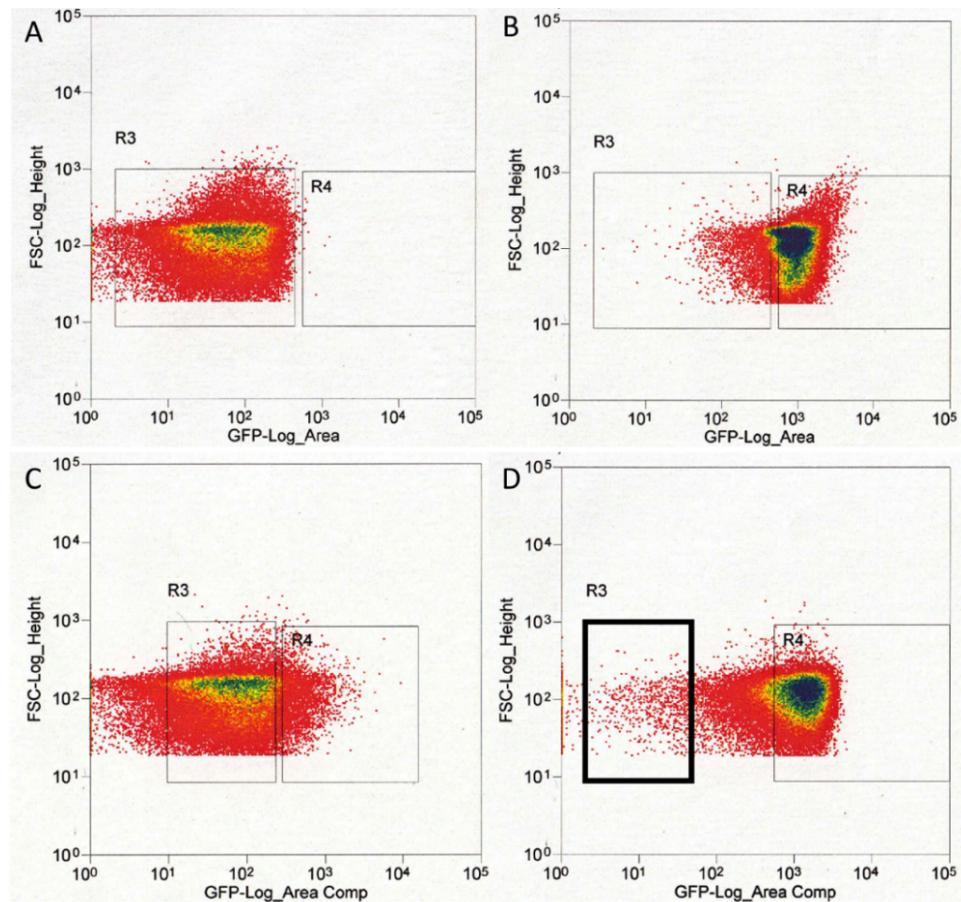
Spray voltage	3.5 kV
Capillary temperature	350 °C
Heater temp	300 °C
S Lens RF level	70 V
Sheath gas flow rate	60
Resolution	70,000
Scan Range	250-1000 m/z



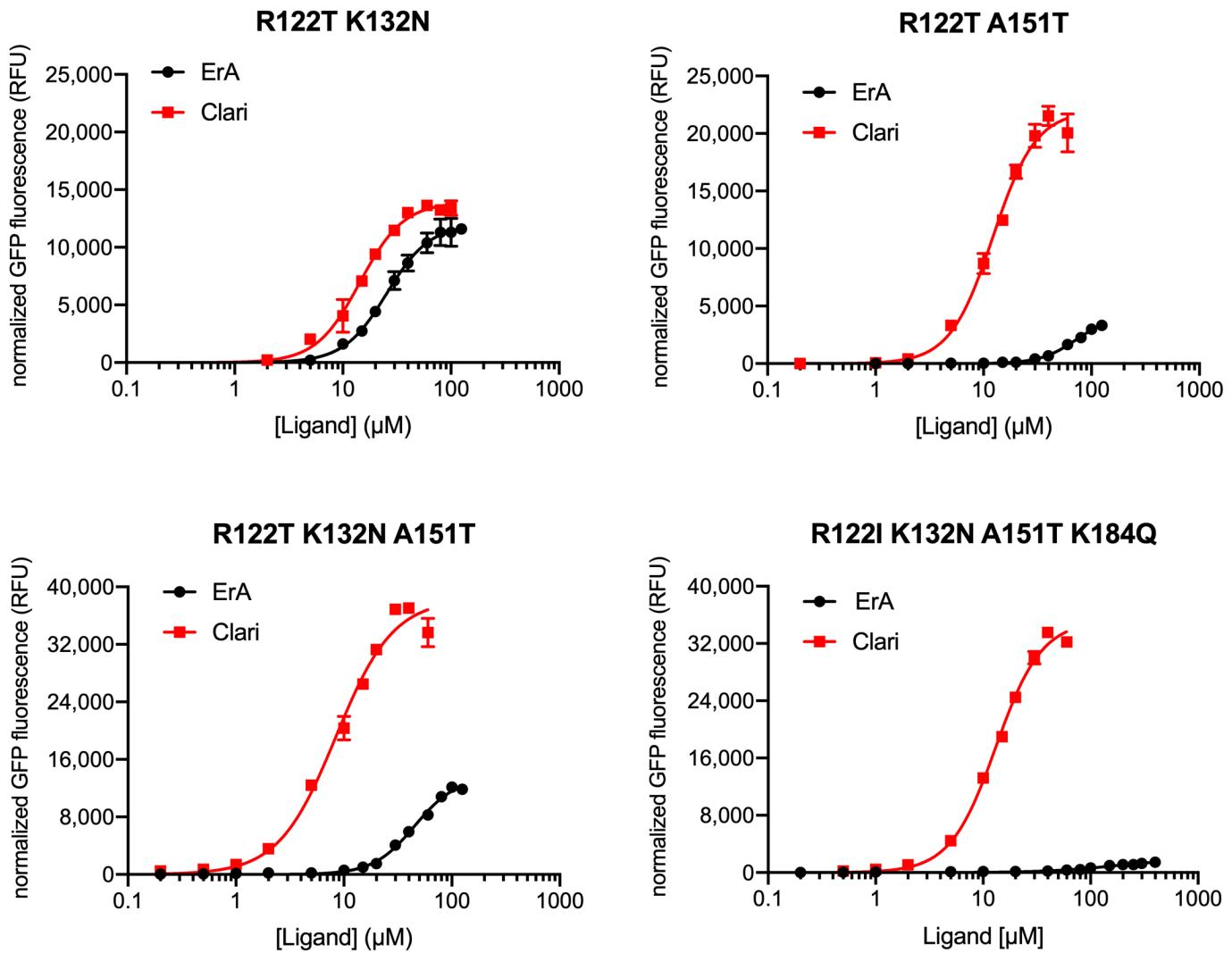
Supplementary Figure S1. Characterization of site-directed MphR biosensor variants. Activities of site-directed mutants using 5 µM ErA, 5 µM CLA, or dimethyl sulfoxide (DMSO). Error-bars represent the standard error of the mean ($n = 3$). Note: A125E in the N123Q/A125E variant is the result of a spurious mutation. N123Q was not tested as a single mutant.



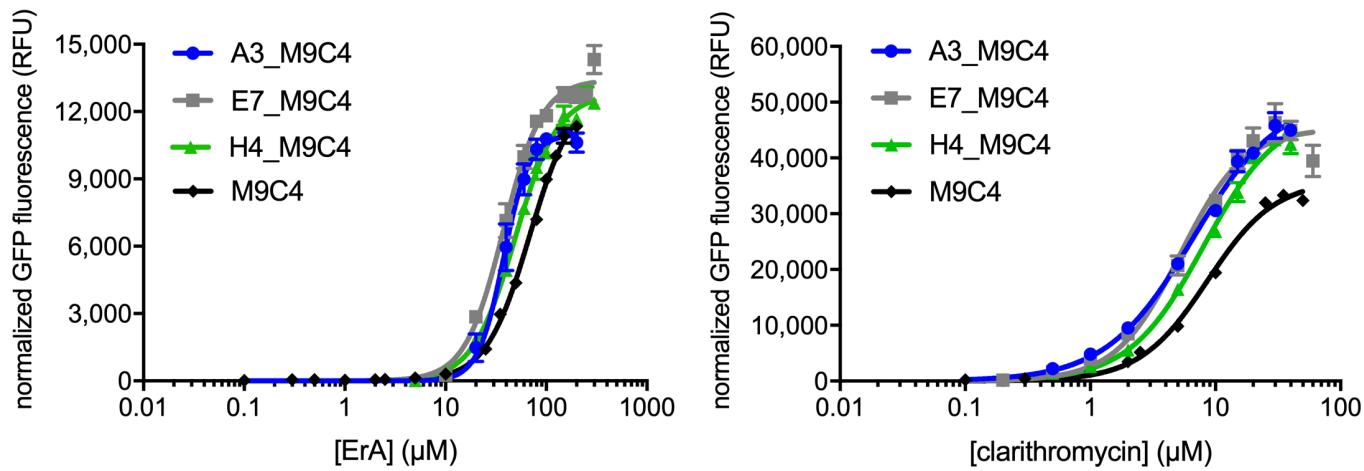
Supplementary Figure S2. The crystal structure of MphR (PDB: 3FRQ) complexed with ErA (green sticks). Locations of Arg122 and Glu109 (blue sticks) that form a salt bridge (dashed lines) are shown.



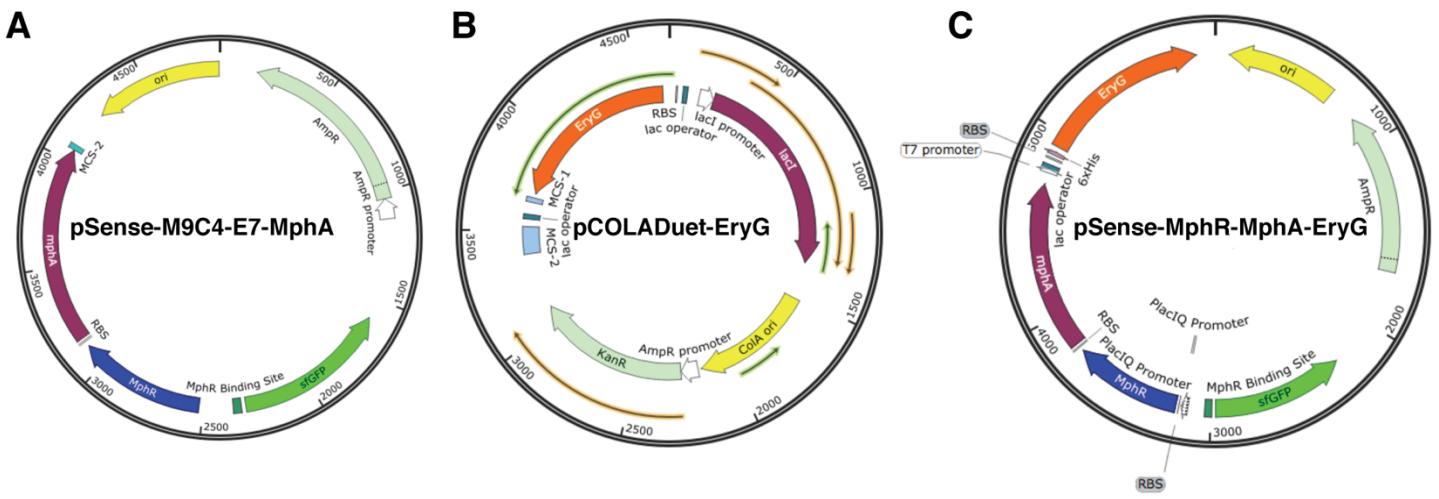
Supplementary Figure S3. FACS analysis and sorting of an error-prone PCR MphR mutant library. (A) Wild-type MphR in the absence of macrolides. (B) Wild-type MphR biosensor strain induced with addition of 5 μ M ErA. (C) MphR mutant library in the absence of macrolides. (D) Induction of the MphR mutant library with addition of 5 μ M ErA. The black box indicates the events corresponding to the ~5% of the library with the lowest fluorescence, corresponding to those variants that were poorly induced with ErA.



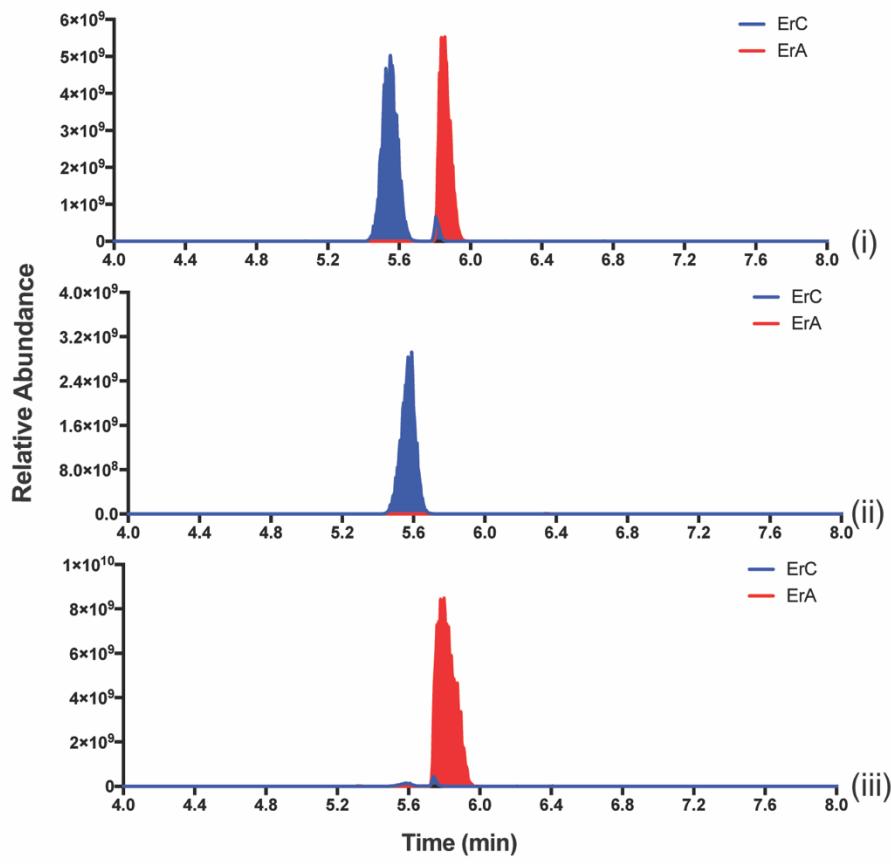
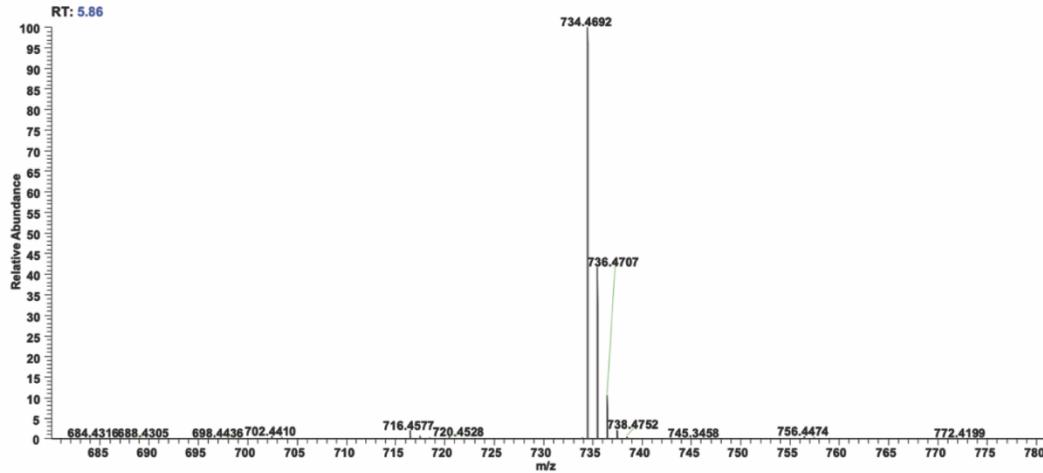
Supplementary Figure S4. Dose-response analysis of the macrolide specificity of mutants derived from M9C4. The biosensor strain is the two-plasmid system consisting of pMLGFP/pJZ12. Error-bars represent the standard error of the mean ($n = 3$) and are only visible when larger than the data point symbol.



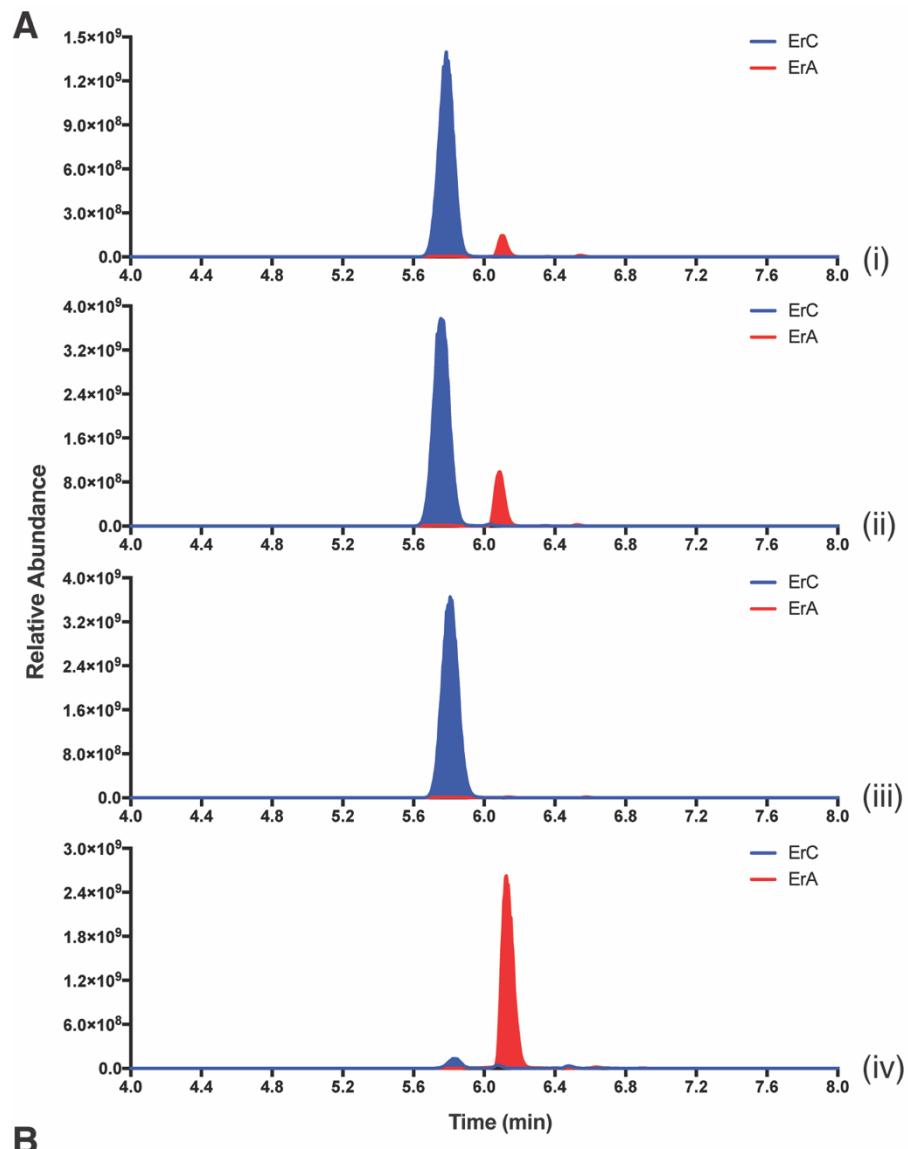
Supplementary Figure S5. Dose-response analysis of RBS mutants of M9C4 MphR. The biosensor strain is the two-plasmid system consisting of pMLGFPK/pJZ12. Error-bars represent the standard error of the mean ($n = 3$) and are only visible when larger than the data point symbol.



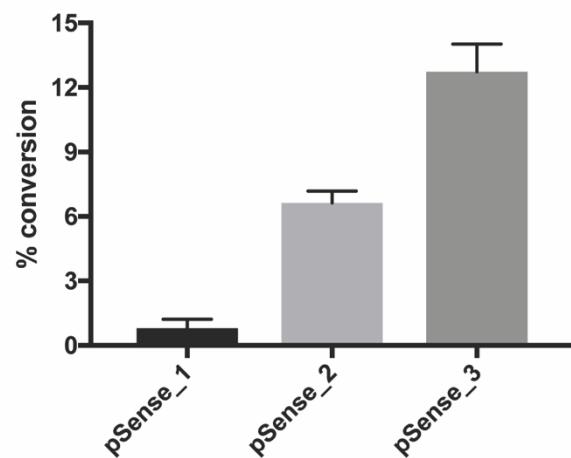
Supplementary Figure S6. Plasmid maps of the various MphR biosensor constructs. (A) pSense-MphR-MphA carries the minimal biosensor system components that the pSense series of plasmids were generated from. (B) pCOLADuet-EryG houses the *O*-MTase gene, EryG. (C) pSense-3 carries the EryG gene and the biosensor components in a single plasmid.

A**B**

Supplementary Figure S7. Representative LC-MS chromatograms of ErA production by *E. coli* BL21(DE3) pCOLADuet-EryG. (A) Extracted ion chromatogram of (i) the *in vivo* conversion of ErC to ErA, (ii) ErC standard, and (iii) ErA standard. Calculated mass for ErA 734.4685 $[M+H]^+$ $[C_{37}H_{68}NO_{13}]^+$; observed mass for ErA standard 734.4689 (Δ , 0.54 ppm). Calculated mass for ErC 720.4529 $[M+H]^+$ $[C_{36}H_{67}NO_{13}]^+$; observed mass for ErC standard 720.4531 (Δ , 0.28 ppm). (B) The representative total ion spectra of ErA produced via *E. coli* BL21(DE3) pCOLADuet-EryG. Observed mass for ErA 734.4692 (Δ , 0.95 ppm)



B



Supplementary Figure S8. ErA production by *E. coli* BL21(DE3) harboring various pSense constructs to determine the requirements for optimal *in vivo* EryG activity. (A) Representative extracted ion chromatogram of (i) ErA production catalyzed by EryG from pSense-2, (ii) ErA production catalyzed by EryG from pSense-3, (iii) ErC standard, (iv) ErA standard. Calculated mass for ErA 734.4685 [M+H]⁺ [C₃₇H₆₈NO₁₃]⁺; observed mass for ErA standard 734.4689 (Δ , 0.54 ppm). Calculated mass for ErC 720.4529 [M+H]⁺ [C₃₆H₆₇NO₁₃]⁺; observed mass for ErC standard 720.4531 (Δ , 0.28 ppm). (B) Percentage conversion of ErC to ErA supported by *E. coli* BL21(DE3) harboring pSense-1 (calculated RBS upstream of EryG, wild-type MphR), pSense-2 (pET28a RBS upstream of EryG, wild-type MphR), and pSense-3 (Entry 14 in **Table 1**; T7 promoter and pET28a RBS upstream of EryG, wild-type MphR). Error-bars represent the standard error of the mean ($n = 3$). Representative observed mass for ErA produced by pSense-3 734.4677 (Δ , -1.1 ppm). Representative observed mass for ErC in pSense-3 sample 720.4515 (Δ , -1.9 ppm).

MPRPKLKSDEVLEAATVVLKRCGPIEFTLSGVAKEVGLSRAALIQRFTNRDTLLVRMMERGVQRHYLNAPI
GAGPQGLWEFLQLVRSMNTRNDFSVNYLISWYELQVPELRTLAIQTNRAVVEGIRNRLPPGAPAAAELLHSVIT
TGATMQWAVIDPDGELADHVLAQIAAILCLMFPEQDDFQLLQAHA

Supplementary Figure S9. Protein sequence of MphR M9C4 used for structural analysis.

SUPPLEMENTARY METHODS

Construction of M9C4 RBS variants and pCOLADuet-EryG

For construction of M9C4-A3, M9C4 -E7, and M9C4 -H4, the pMLGFPK-M9C4 plasmid was used along with the primers listed in **Supplementary Table S3** (entries 37-42). The eryG gene was sub-cloned into pET28a from pET21c-EryG (kindly provided by Dr. Blaine Pfeifer, University of Buffalo). It was then PCR amplified (using primers EryG_PCR_NcoI and EryG_PCR_HindIII, entries 43-44, **Supplementary Table S3**) and sub-cloned into pCOLADuet via the restriction sites *Nco*I and *Hind*III to generate pCOLADuet-EryG.

Construction of pSense_MphR_MphA (used for constructions of pSense-M9C4-MphA-EryG variants)

The pSense-2 plasmid containing *mphO* was ordered as two parts from Twist Bioscience and constructed using Gibson Assembly. MphR and MphA were PCR amplified out of pMLGFP and pJZ12, respectively using primers 45-48 (**Supplementary Table S3**). pSENSE2 was then PCR amplified using primers pSENSE.F and pSENSE.R (entries 49-50, **Supplementary Table S3**) to produce the vector fragment. The PCR products were evaluated using an agarose gel and the resulting products were extracted using an NEB Monarch Gel Extraction Kit. The three fragments were assembled via Gibson Assembly leveraging the NEBuilder Hifi DNA Assembly kit in accordance with the manufacturer's instructions. 2 μ L of the Gibson Assembly were then transformed into *E. coli* Top10 competent cells and the construct was sequenced to confirm successful assembly.

Construction of pSense-M9C4-MphA-EryG variants

An RBS sequence identified via the RBS calculator was embedded into a 50 bp oligonucleotide (entry 51, **Supplementary Table S3**) that was synthesized by Integrated DNA Technologies. The RBS oligonucleotide was incorporated into pSense-MphR-MphA via Gibson Assembly using two primers (entries 52-53, **Supplementary Table S3**), furnishing pSense-MphA-MphR-RBScalculate. The sequencing data indicated 2 and 20 base pairs were randomly inserted before and after the 50-base oligonucleotide region in pSense-MphAR-RBScalculate but they are not expected to impact transcription/translation. The eryG gene was then amplified from pET28a-EryG and sub-cloned into pSense-MphA-MphR-RBScalculate via *Nde*I and *Kpn*I restriction sites to generate ‘pSense-1’ using primers 54-55 (**Supplementary Table S3**). To generate ‘pSense-2’, the calculated RBS from pSense-1 was substituted with the RBS from pET28a via Gibson Assembly using primers 56-57 and 59-60 (**Supplementary Table S3**). To generate ‘pSense-3’, the promoter in pSense-1 was substituted by the T7 promoter via Gibson Assembly using primers 57-59 and 61 (**Supplementary Table S3**). To generate ‘pSense-M9C4-E7-MphA-EryG’ (relevant to **Figure 5B-D**), wild-type MphR in pSense-3 was substituted with M9C4-E7 (amplified from pMLGFPK-E7M9C4) via Gibson Assembly using primers 62-65 (**Supplementary Table S3**).

In vivo assay of EryG activity

A 10 mL culture of BL21(DE3) containing the desired plasmids was grown in LB media supplemented with appropriate antibiotics at 37 °C with shaking at 250 rpm. The corresponding antibiotics for three-plasmid, two-plasmid and one-plasmid systems were ampicillin 67 μ g/mL, tetracycline 3 μ g/mL, kanamycin 33 μ g/mL; ampicillin 67 μ g/mL, kanamycin 33 μ g/mL; and ampicillin 100 μ g/mL. Cultures were grown to an OD₆₀₀ ~0.6 at which point 50 μ M ErC and 0.5 mM IPTG was added.

After incubating the cell culture at 37 °C and 250 rpm overnight, 1.5 mL of culture was withdrawn and centrifuged for 30 min at 10,000 g. Then, 500 µL of the supernatant was extracted three times with an equal volume of ethyl acetate and dried. The dried extract was resuspended in 200 µL of methanol for LC-HRMS analysis.

LC-HRMS analysis of ErA production

LC-HRMS analysis was performed by the NC State the Molecular Education, Technology and Research Innovation Center using a Thermo Fisher Scientific Exactive Plus mass spectrometer and Heated Electrospray Ionization (HESI). The sample was analyzed via LC-MS injection into the mass spectrometer at a flow rate of 500 µL/min. The mobile phase B was acetonitrile with 0.1% formic acid and mobile phase A was water with 0.1% formic acid. See **Supplementary Table S5** for the gradient and HESI source parameters. The mass spectrometer was operated in positive ion mode. The LC column was a Thermo Hypersil Gold 50 x 2.1 mm, 1.9 µm particle size. This assay produces ErA and ErC that can be seen as their $[M+H]^+$ ions. Calculated mass for ErA 734.4685 $[M+H]^+ [C_{37}H_{68}NO_{13}]^+$; representative observed mass for ErA 734.4689 (Δ , 0.54 ppm). Calculated mass for ErC 720.4529 $[M+H]^+ [C_{36}H_{67}NO_{13}]^+$; representative observed mass for ErC 720.4531 (Δ , 0.28 ppm). In every case, the error ($\Delta = [\text{Difference/exact mass} \times 10^6]$) was < 6 ppm.