

Multicopy chromosome integration and deletion of negative global regulators significantly increased the heterologous production of aborycin in *Streptomyces coelicolor*

Supplementary Figures and Tables

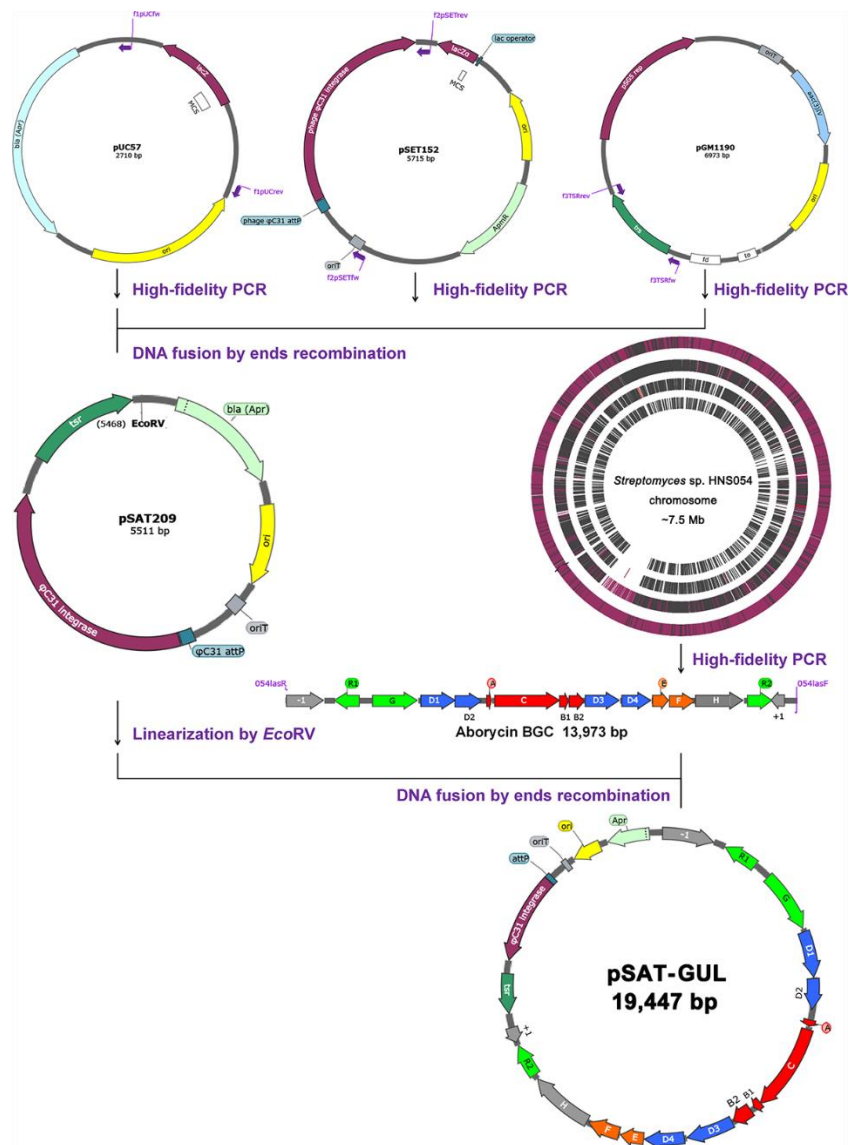


Figure S1 Construction of the integrative vector for aborycin expression. The f1 fragment (*bla*+*ori*^{pUC}) was amplified from the pUC57 plasmid by primers f1pUCfw and f1pUCrev. The f2 fragment (*oriT*+*attP*+*int*) was amplified from the pSET152 plasmid by primers f2pSETfw and f2pSETRev. And the f3 fragment (*tsr*) was amplified from the pGM1190 plasmid by primers f3TSRfw and f3TSRrev2. Then the three fragments were fused into the integrated plasmid

pSAT209 by using the Multi-fragment Assembly Kit (CAT # C113, Vazyme, Nanjing, China). By using the same assembly kit, the *EcoRV*-linearized pSAT209 was fused with the 14 Kb aborycin BGC fragment, which was amplified from the genomic DNA of the HNS054 strain with primers 054LasF and 054LasR, to form the final integrative vector pSAT-GUL. Colonies were screened by PCR with primers gul-cF and gul-cR. The positive pSAT-GUL plasmid was whole sequenced to confirm sequence accuracy. All high-fidelity PCR reactions were performed by using the DNA polymerase 2×Phanta Max Kit (CAT # P515, Vazyme, Nanjing, China).

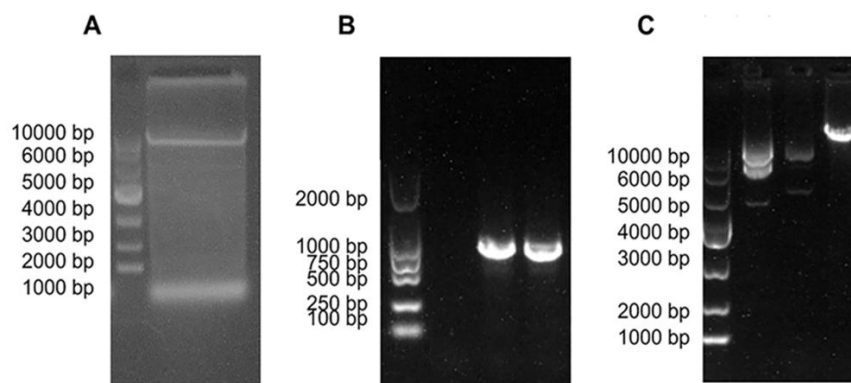


Figure S2 Clone of the *gul* gene cluster. (A) **Amplification of *gul* gene cluster.** From left to right, DNA marker and the PCR fragment of *gul* gene cluster. (B) **Screening of vector pSAT-GUL.** From left to right, DNA marker, PCR fragments with primers gul-cF and gul-cR from blank control and two positive pSAT-GUL colonies. (C) **Plasmids.** From left to right, DNA marker, plasmid pSAT209, pSET152 and pSAT-GUL.

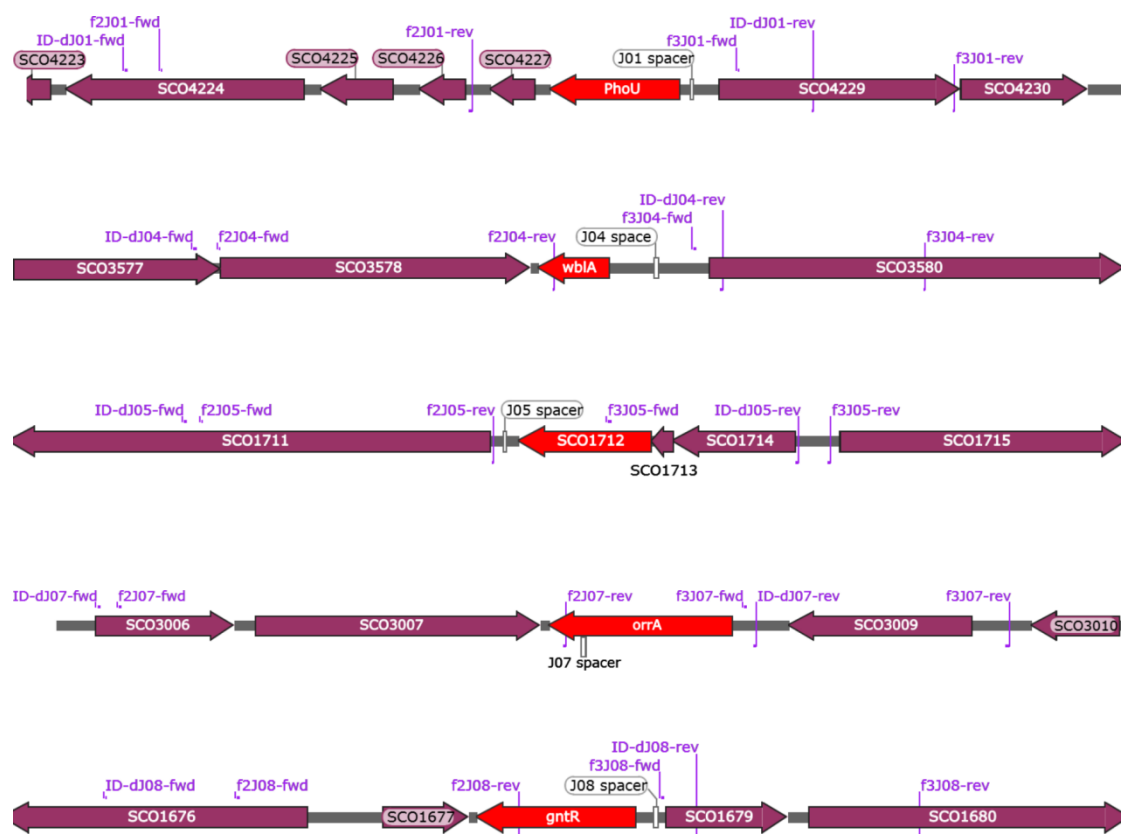


Figure S3 CRISPR/Cas9 constructions of the five gene-knockout mutants from M1346::3*gul*. Gene organizations, spacer positions and primers for deletion of *phoU*, *wblA*, *SCO1712*, *orrA* and *gntR* genes, respectively.

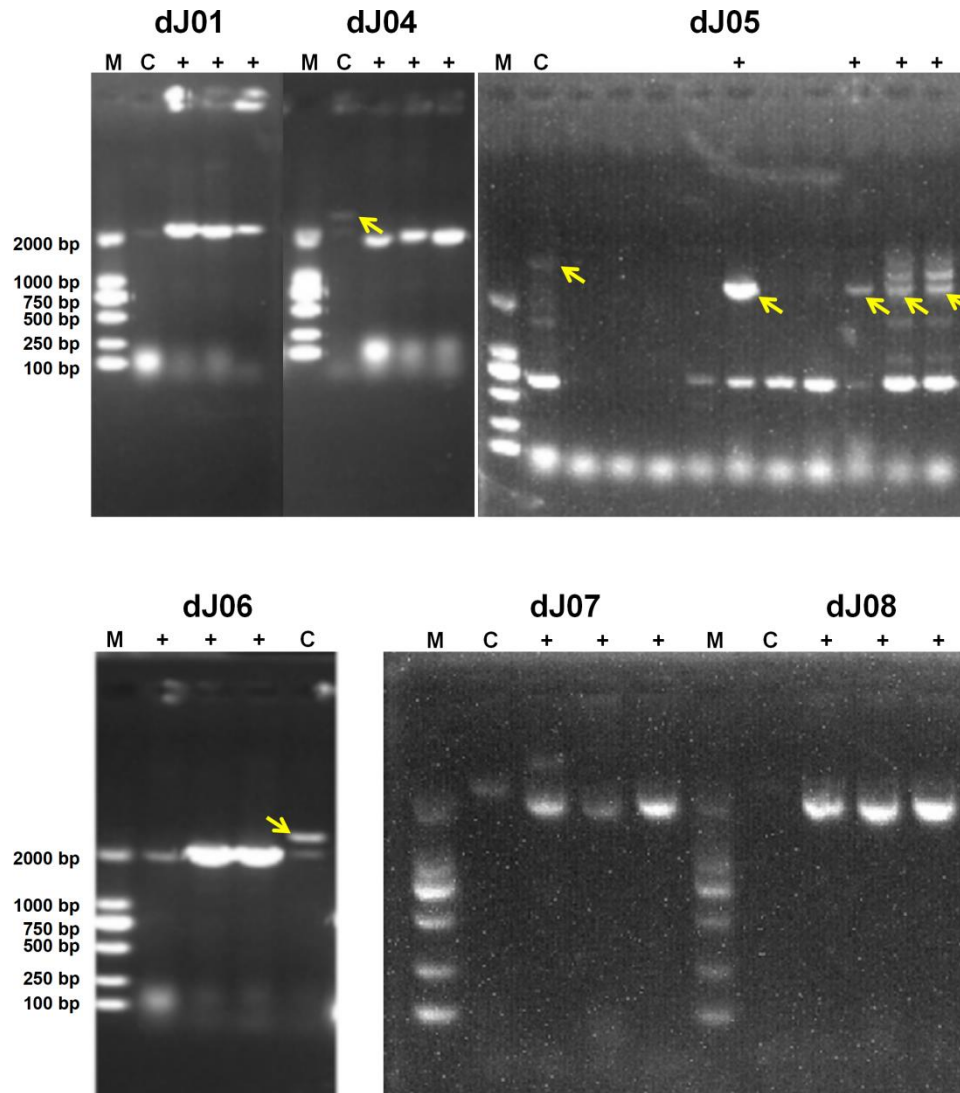


Figure S4 PCR screening for positive mutants. d01 - d08 shows screening for $\Delta phoU$, $\Delta wblA$, $\Delta SCO1712$, $\Delta orrA$ and $\Delta gntR$ mutants of M1346::3gul, respectively. C, the control strain M1346::3gul. M, DNA marker. +, positive exconjugant. Yellow arrow, to indicate the correct band. All correct bands from the positive exconjugants were sequenced to confirm the success of deletion.

Table S1 Information of plasmids and strains

Plasmids	Relevant features	Source/Reference
pGM1190	<i>tsr, rep^{SG5}, oriT, acc(3)IV, ori^{pUC}</i>	[1]
pSET152	<i>oriT, attP_{ΦC31}, int_{ΦC31}, lacZα, ori^{pUC}, acc(3)IV</i>	[2]
pUC57	<i>bla, ori^{pUC}, lacZα</i>	Thermo Scientific
pSAT209	<i>tsr, oriT, attP_{ΦC31}, int_{ΦC31}, bla, ori^{pUC}</i>	This study
pSAT-GUL	<i>tsr, oriT, attP_{ΦC31}, int_{ΦC31}, bla, ori^{pUC}, gul</i>	This study
pKCCas9	<i>acc(3)IV, ori^{pUC}, rep^{SG5}, oriT, tipA-Scocas9</i>	[3]
pKCCas9dO	<i>acc(3)IV, ori^{pUC}, rep^{SG5}, oriT, tipA-Scocas9, j23119-actII-orf4-gRNA, homologous region flanking actII-orf4</i>	[3]
pKCCas9-dJ01	<i>acc(3)IV, ori^{pUC}, rep^{SG5}, oriT, tipA-Scocas9, j23119-J01spacer-gRNA, homologous region flanking phoU</i>	This study
pKCCas9-dJ04	<i>acc(3)IV, ori^{pUC}, rep^{SG5}, oriT, tipA-Scocas9, j23119-J04spacer-gRNA, homologous region flanking wblA</i>	This study
pKCCas9-dJ05	<i>acc(3)IV, ori^{pUC}, rep^{SG5}, oriT, tipA-Scocas9, j23119-J05spacer-gRNA, homologous region flanking SCO1712</i>	This study
pKCCas9-dJ07	<i>acc(3)IV, ori^{pUC}, rep^{SG5}, oriT, tipA-Scocas9, j23119-J07spacer-gRNA, homologous region flanking orrA</i>	This study
pKCCas9-dJ08	<i>acc(3)IV, ori^{pUC}, rep^{SG5}, oriT, tipA-Scocas9, j23119-J08spacer-gRNA, homologous region flanking gntR</i>	This study
Strains	Relevant features	Source/Reference
<i>E.coli</i> ET12567(pUZ8002)	<i>dam, dcm, hsdS, cat, tet, tra, neo, RP4</i>	[4]
<i>Streptomyces</i> sp. HNS054	Source strain of the aborycin gene cluster <i>glu</i>	[5]
<i>S.coelicolor</i> M1146	<i>S. coelicolor</i> M145 (contains one native <i>attB_{ΦC31}</i>) Δact , Δred , Δcpk , Δcda	[6]

<i>S.coelicolor</i> M1246	<i>S. coelicolor</i> M1146 $\Delta cpk::attB_{\Phi C31}$	[7]
<i>S.coelicolor</i> M1346	<i>S. coelicolor</i> M1246 $\Delta red::attB_{\Phi C31}$	
<i>S.coelicolor</i> M1446	<i>S.coelicolor</i> M1346 $\Delta cda::attB_{\Phi C31}$	
<i>S.coelicolor</i> M1546	<i>S.coelicolor</i> M1446 $\Delta act::attB_{\Phi C31}$	
M1146:: pSAT209	<i>S.coelicolor</i> M1146 integrates one copy of pSAT209 at the native <i>attB</i> _{$\Phi C31$}	This study
M1146::gul	<i>S.coelicolor</i> M1146 integrates one copy of pSAT-GUL at the native <i>attB</i> _{$\Phi C31$}	This study
M1246::2gul	<i>S.coelicolor</i> M1246 integrates two copy of pSAT-GUL at two <i>attB</i> _{$\Phi C31$} sites	This study
M1346::3gul	<i>S.coelicolor</i> M1346 integrates three copy of pSAT-GUL at three <i>attB</i> _{$\Phi C31$} sites	This study
M1346::3gul $\Delta phoU$	<i>S.coelicolor</i> M1346::3gul without the <i>phoU</i> gene	This study
M1346::3gul $\Delta wblA$	<i>S.coelicolor</i> M1346::3gul without the <i>wblA</i> gene	This study
M1346::3gul $\Delta SCO1712$	<i>S.coelicolor</i> M1346::3gul without the <i>SCO1712</i> gene	This study
M1346::3gul $\Delta orrA$	<i>S.coelicolor</i> M1346::3gul without the <i>orrA</i> gene	This study
M1346::3gul $\Delta gntR$	<i>S.coelicolor</i> M1346::3gul without the <i>gntR</i> gene	This study

Table S2 Primer sequence used in this study

Primers	Sequence (5'-3')
054lasF	cgattcgtcagtgatgatcTCCGTTGGCAAGGTTTGATGTG
054lasR	aaggatccactagtgatcACGACGAGAAGGAGACCGAGG C
f1pUCfw	gaaatggACCGTCATCACCGAAAC
f1pUCrev	gccaaccaATAACGCAGGAAAGAACAT
f2pSETfw	gcgttatTGGTTGGCTTGGTTTCAT
f2pSETrev	ccgctctCACGCCCTCCTACATCG
f3TSRfw	agggcgtgAGAGCGGGGAGCTTTG
f3TSRrev	atgacggtCCATTTCTGGGTCCTGTTG
gul-cF	TGTCTATCGCTCCTTCGTTTCCA
gul-cR	GTCTCCCTGCTCTTCACCTCGTT
ID-oriT-fw	gcagagcaggattcccggttgagca
ID-native-attB-rev	acgtcccgtgctcaccgtgacca

ID-CPK-attB-rev		cccatgctgtgccgaagaa
ID-RED-attB-rev		tccagcgcttggtggcgtagcgtt
ID-CDA-attB-rev		catgacgcaacgcaagaagagct
ID-ACT-attB-rev		tggtcaccgtctccatgggtcatgt
f1gRNA-R		gtggaattcagatctcaaaaa
J01 set	f1J01-fwd	agtcctaggtataataactagtGCCCCCACGCTTACACGTTC gttttagagctagaaatagca
	f2J01-fwd	ttttgagatctgaattccacAGGAACTGCGGGGTGGA
	f2J01-rev	CTGGAAGGAGGACCCTGAAG
	f3J01-fwd	cttcagggtctctctccagGAGCGGGAGCAGAAGCG
	f3J01-rev	acgacggccagtgccaagcttCGAACGGGGCTGTATGGG
	ID-dJ01-fwd	ccgttggtgtccagaagc
	ID-dJ01-rev	cagctcgtggctcacgttg
J04 set	f1J04-fwd	agtcctaggtataataactagtTCATCTCTGTTGCGCCACAAG tttttagagctagaaatagca
	f2J04-fwd	ttttgagatctgaattccacTGCGGAAGCAGGGGATC
	f2J04-rev	GCGGACGGAGTACGAACG
	f3J04-fwd	CTCGTTCGTA CTCCGTCCTACTGCTGTGACA GTTGAGTGC
	f3J04-rev	acgacggccagtgccaagcttAGCGTCGTGCGGATGG
	ID-dJ04-fwd	CGCTGGAGCAGGAGCAG
	ID-dJ04-rev	TGACACCGAGGAACTTGGC
J05 set	f1J05-fwd	agtcctaggtataataactagtCGGTAGGCATGTTGGGGAGG gttttagagctagaaatagca
	f2J05-fwd	gaattccacGTGTTGAAGGCGAAGTCGTGG
	f2J05-rev	aacaagcgccagatggTTCCGGGAGGACCCGTGC
	f3J05-fwd	ggaaCCATCTGGCGCTTGTGTC
	f3J05-rev	acgacggccagtgccaagcttCGTAGGATTGCGGTTTCCC
	ID-dJ05-fwd	ATCTCCGTCTCGGCCATCCTG
	ID-dJ05-rev	CCCGTGTCTAGTCTCGCGTCAT
J07 set	f1J07-fwd	agtcctaggtataataactagtGTCCCGTTGTTTCATTCCCGg tttttagagctagaaatagca
	f2J07-fwd	ttttgagatctgaattccacGGACCCCGACATCCAACG
	f2J07-rev	GGAGAAACTCCAGCTGCACTC
	f3J07-fwd	gagtgacagctggagtttctcCGCCAACCCAGAGCAACGA
	f3J07-rev	acgacggccagtgccaagcttGCGAGATATGTTCCGTTGTG AG
	ID-dJ07-fwd	AACCCGTCACGCTCACC
	ID-dJ07-rev	GGGCATGGAATCATGGC
J08 set	f1J08-fwd	agtcctaggtataataactagtGTTTCAGTTGCGTCGACATCg tttttagagctagaaatagca
	f2J08-fwd	ttttgagatctgaattccacGTGGGTCCAGTCGTCCG
	f2J08-rev	GTCGTCGCCCAGGTCC

f3J08-fwd	gccaggacctcggcgacgacCGACGAAGACAGCAGACA GC
f3J08-rev	acgacggccagtgccagcttCCGCATCAGCGAGTAGTTG
ID-dJ08-fwd	GGTGTTCGCTCAGGGTGGC
ID-dJ08-rev	CGGAGGGTGGAAGTCGTC

Table S3 The length of RiPP biosynthetic gene clusters

Class/example	Length	Reference
Proteusins	13.6kb	[8]
Lasso peptides		[9]
MS-271	14kb	
(siamycin)		
specialicin	14kb	
Thiopeptides		[10]
thiomuracin	13.4kb	
berninamycin	11kb	
lactazole	8.4kb	
Linaridins		[11]
cypemycin	8.5kb	
legonaridin	8.5kb	
Cyanobactins	<10kb	[12]
Bottromycins	17kb	[13]
Microviridin	6.8kb	[14]
Glycocins	4kb	[15]
Microcins		[16]
Class I	4-5.5kb	
Class II	3.5-4kb	
Class III	8.5-11.5kb	

Table S4 RiPP examples reported by Montalbán-López et al. [17]

Class/example	length
Crocagins/Crocagin A	13kb
<i>Sulfatytrotides/RaxX</i>	4kb
Darobactin	7kb
Tryptorubin A	4kb
Cacaoidin	28kb
Cittilins/Cittilin A	1.9kb
<i>Lipolanthines/Microvionin</i>	23kb
Thiopeptide/Thiomuracin	13kb

Linaridin/Cypemycin	13kb
LAP /Microcin B17	6kb
Thioamitide/Thioholgamide	10kb
<i>Thioamitides/Thioviridamide</i>	11kb
Cyanobactin /Patellamide	13kb
Bottromycins /Bottromycin A1	18kb
Lasso peptide/Microcin J25	6kb
Sactipeptide/Subtilosin A	8kb
Ranthipeptide/Freyrasin	4kb
<i>Streptide</i> /Streptide	4kb
Epipeptides/Yyd	5kb
Spliceotides/Plp	12kb
Graspetide/Microviridin J	3kb
Dikaritins/Ustiloxin	20kb
Asperipin 2a	5kb
Phomopsins	20kb
Pheganomycin	28kb
Pyrroloquinoline quinones/PQQ	9kb
<i>Mycofactocin/Mycofactocin</i>	15kb
Methanobactins/Methanobactin	13kb
3-thiaglutamate	15kb
Aeruginosamide B	15kb
Landornamide	14kb
Goadsporin	23kb
Thiocillin	16kb

Supplementary references

1. Muth, G.; Nußbaumer, B.; Wohlleben, W.; Pühler, A. A vector system with temperature-sensitive replication for gene disruption and mutational cloning in *Streptomyces*. *Molecular and General Genetics MGG* **1989**, 219, 341-348, doi:10.1007/BF00259605.
2. Bierman, M.; Logan, R.; O'Brien, K.; Seno, E.T.; Rao, R.N.; Schonher, B.E. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **1992**, 116, 43-49, doi:10.1016/0378-1119(92)90627-2.
3. Huang, H.; Zheng, G.; Jiang, W.; Hu, H.; Lu, Y. One-step high-efficiency CRISPR/Cas9-mediated genome editing in *Streptomyces*. *Acta biochimica et biophysica Sinica* **2015**, 47, 231-243, doi:10.1093/abbs/gmv007.
4. MacNeil, D.J.; Gewain, K.M.; Ruby, C.L.; Dezeny, G.; Gibbons, P.H.; MacNeil, T. Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration

- vector. *Gene* **1992**, *111*, 61-68, doi:10.1016/0378-1119(92)90603-m.
5. Liu, T.; Huang, Z.; Gui, X.; Xiang, W.; Jin, Y.; Chen, J.; Zhao, J. Multi-omics comparative analysis of *Streptomyces* mutants obtained by iterative atmosphere and room-temperature plasma mutagenesis. *Frontiers in microbiology* **2020**, *11*, 630309, doi:10.3389/fmicb.2020.630309.
 6. Gomez-Escribano, J.P.; Bibb, M.J. Engineering *Streptomyces coelicolor* for heterologous expression of secondary metabolite gene clusters. *Microbial biotechnology* **2011**, *4*, 207-215.
 7. Li, L.; Zheng, G.; Chen, J.; Ge, M.; Jiang, W.; Lu, Y. Multiplexed site-specific genome engineering for overproducing bioactive secondary metabolites in actinomycetes. *Metabolic engineering* **2017**, *40*, 80-92, doi:10.1016/j.ymben.2017.01.004.
 8. Arnison, P.G.; Bibb, M.J.; Bierbaum, G.; Bowers, A.A.; Bugni, T.S.; Bulaj, G.; Camarero, J.A.; Campopiano, D.J.; Challis, G.L.; Clardy, J.; et al. Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Natural product reports* **2013**, *30*, 108-160, doi:10.1039/c2np20085f.
 9. Kaweewan, I.; Hemmi, H.; Komaki, H.; Harada, S.; Kodani, S. Isolation and structure determination of a new lasso peptide specialicin based on genome mining. *Bioorg Med Chem* **2018**, *26*, 6050-6055, doi:10.1016/j.bmc.2018.11.007.
 10. Vinogradov, A.A.; Suga, H. Introduction to thiopeptides: biological activity, biosynthesis, and strategies for functional reprogramming. *Cell chemical biology* **2020**, *27*, 1032-1051, doi:10.1016/j.chembiol.2020.07.003.
 11. Georgiou, M.A.; Dommaraju, S.R.; Guo, X.; Mast, D.H.; Mitchell, D.A. Bioinformatic and Reactivity-Based Discovery of Linaridins. *ACS chemical biology* **2020**, *15*, 2976-2985, doi:10.1021/acscchembio.0c00620.
 12. Sivonen, K.; Leikoski, N.; Fewer, D.P.; Jokela, J. Cyanobactins-ribosomal cyclic peptides produced by cyanobacteria. *Applied microbiology and biotechnology* **2010**, *86*, 1213-1225, doi:10.1007/s00253-010-2482-x.
 13. Franz, L.; Kazmaier, U.; Truman, A.W.; Koehnke, J. Bottromycins - biosynthesis, synthesis and activity. *Natural product reports* **2021**, *38*, 1659-1683, doi:10.1039/d0np00097c.
 14. do Amaral, S.C.; Monteiro, P.R.; Neto, J.; Serra, G.M.; Gonçalves, E.C.; Xavier, L.P.; Santos, A.V. Current knowledge on microviridin from Cyanobacteria. *Marine Drugs* **2021**, *19*, doi:10.3390/md19010017.
 15. Singh, V.; Rao, A. Distribution and diversity of glycoicin biosynthesis gene clusters beyond Firmicutes. *Glycobiology* **2021**, *31*, 89-102, doi:10.1093/glycob/cwaa061.
 16. Duquesne, S.; Destoumieux-Garzón, D.; Peduzzi, J.; Rebuffat, S. Microcins, gene-encoded antibacterial peptides from enterobacteria. *Natural product reports* **2007**, *24*, 708-734, doi:10.1039/b516237h.
 17. Montalbán-López, M.; Scott, T.A.; Ramesh, S.; Rahman, I.R.; van Heel, A.J.; Viel, J.H.; Bandarian, V.; Dittmann, E.; Genilloud, O.; Goto, Y.; et al. New developments in RiPP discovery, enzymology and engineering. *Natural product reports* **2021**, *38*, 130-239, doi:10.1039/d0np00027b.