

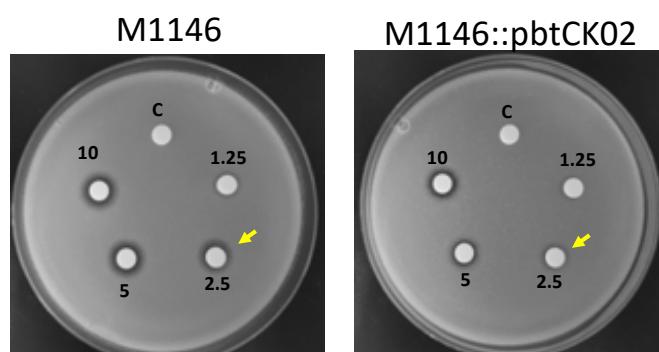
1 Engineering *Streptomyces coelicolor* for heterologous
2 expression of the thiopeptide GE2270A – a cautionary
3 tale

4

5 **Supplementary material**

6 **Attempts to improve GE2270A tolerance of *S. coelicolor* thorough the overexpression of different
7 resistant versions of EF-Tu protein**

8 The first GE2270A heterologous producer, *S. coelicolor* M1146::pbtCK02 (LW221) is still sensitive
9 to GE2270A (Flinspach et al., 2014) despite possessing the resistant gene, *Prtuf* (*tuf* gene from the
10 natural producer *Planobisporaea rosea*), under the control of the constitutive promoter, *ermE**p. We
11 reproduced these results during the optimisation of the GE2270 production conditions, when agar
12 diffusion bioassays of cell extracts only showed a very slight improvement, if any, in GE2270A
13 tolerance relative to the parental strain (Fig. S1). This very low level of tolerance is not likely to
14 protect any future GE2270A-overproducer strain, and we set out to find ways to increase it.



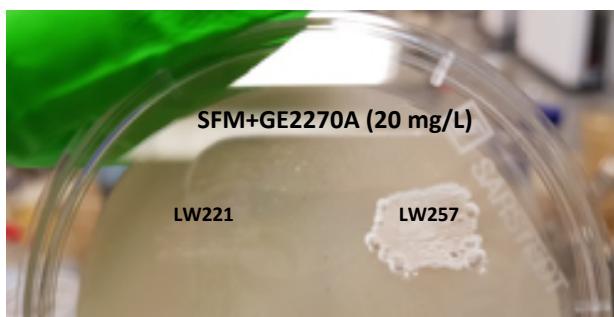
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16 **Figure S1. Agar diffusion bioassays of GE2270A tolerance by M1146 and M1146::pbtCK02.** Numbers
17 indicate amounts of pure GE2270A in µg. The arrow depicts the minimal amount of GE2270A in which
18 bioactivity is detected.

19

20 To increase GE2270A tolerance by M1146, the overexpression of different resistant versions of the
21 EF-Tu protein were attempted: *P. rosea* tuf (*Prtuf*); a mutated version (*muttufl*) of *S. coelicolor*'s *tuf1*
22 containing 4 point mutations present in *Prtuf* (G257S, V274A, G275A, V291C); and a secondary EF-
23 Tu coding gene *tuf3* present in *S. coelicolor*'s genome (*Sctuf3*).

24 Over-expression of *Planobispora rosea* tuf (*Prtuf*)

25 As discussed above, over-expression of *Prtuf* using the strong *ermE**p promoter has already been
26 attempted to negligible effects¹ (**Fig S1**). We reasoned that, when both *P. rosea* and *S. coelicolor* EF-
27 Tu are present, the translation machinery of *S. coelicolor* might largely disregard the heterologous,
28 albeit resistant version, and use preferentially its cognate, though sensitive EF-Tu. This would indeed
29 result in cells that are still sensitive to GE2270A. Based on this hypothesis, one of the options we
30 have explored was to replace the *Sctuf1* on the M1146 chromosome with *Prtuf*, in an attempt to force
31 the strain to use the resistant variant. To do so, we decided to implement the CRISPR-Cas9 system²,
32 obtaining strain LW257. As shown in **Fig. S2**, LW257 resistance against GE2270A was tested by
33 plating it on SFM + GE2270A (20 mg/L). Parental strain LW221 was also grew as control.



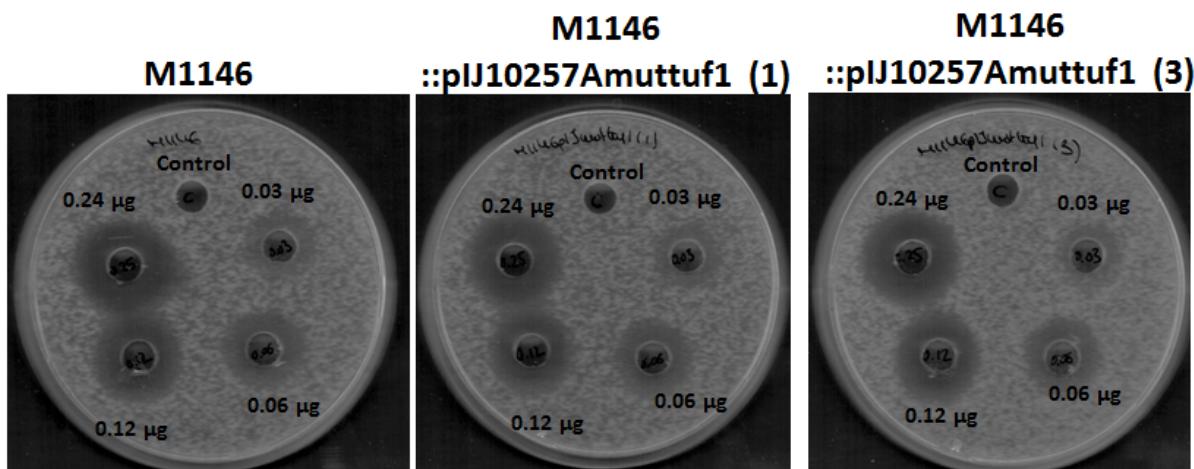
34
35 **Figure S2. Bioassay testing resistance to GE2270A of strains LW221 and LW257.**

36 While this approach was successful in conferring resistance, it severely impacted *S. coelicolor*
37 growth, making the obtained strain unsuitable for future developments.

38 Expression of a mutated version of *Streptomyces coelicolor* *tuf1*

39 Another way to engineer resistance via EF-Tu is to modify *S. coelicolor* EF-Tu amino acid sequence
40 to that of *P. rosea*. Therefore, a mutated version of the *Sctuf1* was synthesised, in which 4 point
41 mutations were introduced (G257S, V274A, G275A, V291C), all of them present in *Prtuf* and thought
42 to be involved in its GE2270A-resistant phenotype and not present in *S. coelicolor*'s *tuf1*. This

43 synthetic construct was ordered as a 2 kb length gBlock to IDT Technologies (Ref. 74028784) and
44 cloned into pCR-Blunt, to generate the construct pBluntnuttuf1. This mutated gene was used both for
45 gene replacement using CRISPR-Cas9 and for ectopic integration at a different site on the genome.
46 For ectopic integration, pIJ1025Amuttuf1 construct was generated, which is a BT1 integrative vector
47 with muttuf1 under the control of the natural *Sctuf1* promoter. This construct was introduced into
48 M1146 by intergeneric conjugation into the phiC31 integration site, generating the strain
49 M1146::pIJ1025Amuttuf1. Two independent colonies, M1146::pIJ1025Amuttuf1 (1) and (3), were
50 tested for their resistance to GE2270A, which was measured by agar diffusion bioassays using
51 *Bacillus subtilis* 168 as indicator strain. Known concentrations of pure GE2270A were inoculated into
52 wells made in the agar, and the plates were incubated for 48h at 30°C. M1146 was used as a control.
53 No differences were observed between the strains (Fig. S3), which may be due to the mutant EF-Tu
54 being a poor functional replacement for WT EF-Tu.



56 **Figure S3. GE2270A tolerance testing of a refactored strains expressing a mutant allele of *Sctuf1* as**
57 **indicated above panels.** Numbers denote given concentrations of pure GE2270A.

58 Over-expression of *Streptomyces coelicolor tuf3*

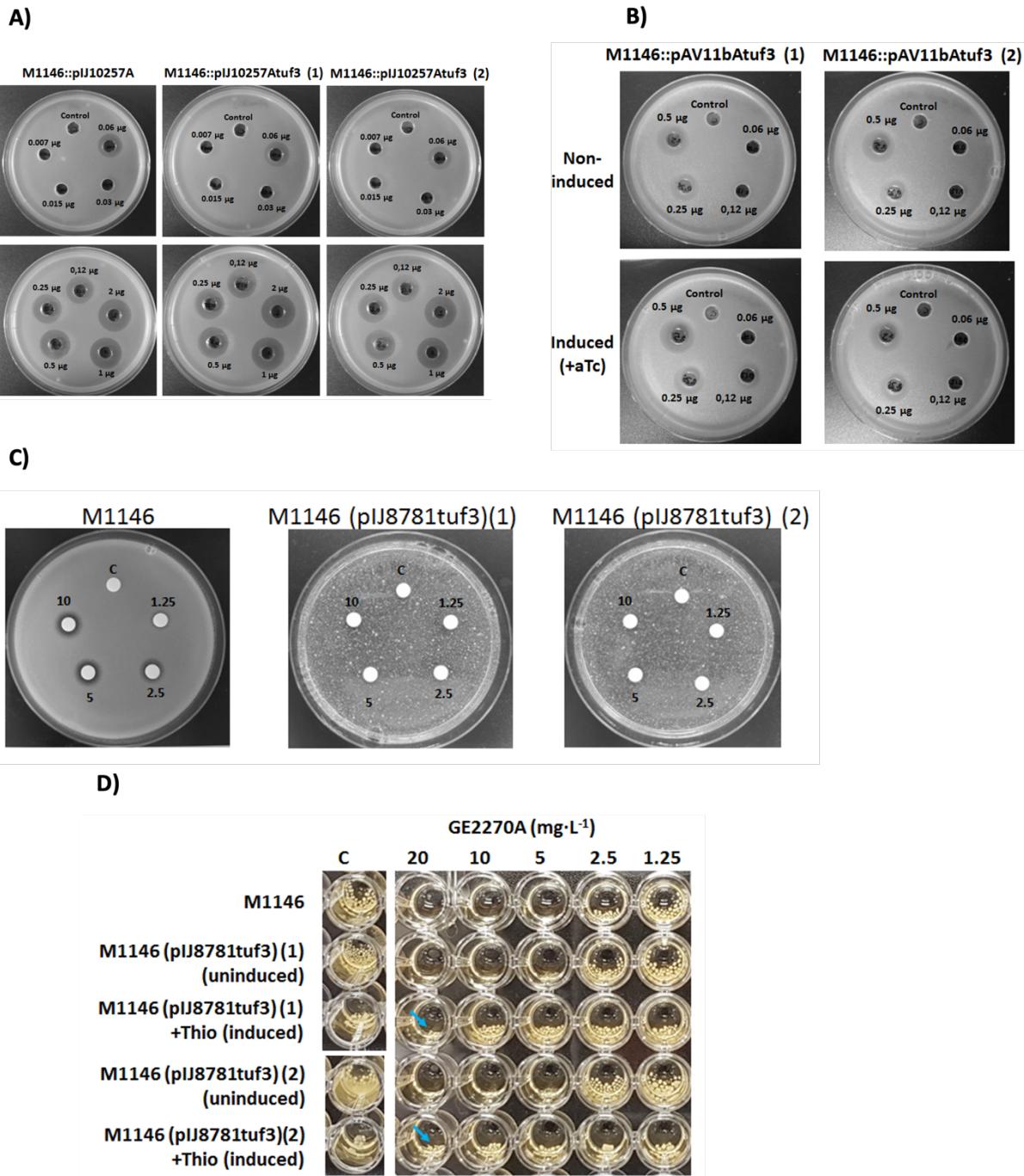
59 Unlike *P. rosea*, which only has one *tuf* gene, many Streptomycetes present two or even three
60 different *tuf* genes. In fact, *S. coelicolor* possess two: *Sctuf1*, coding for the house-keeping EF-Tu,
61 which is GE2270A-sensitive and has been discussed above, and *Sctuf3*, which is expressed only under

62 stress conditions (e.g., amino acid starvation)³, and codes for a protein that, albeit less active than EF-
63 Tu1, is instead GE2270A-resistant⁴.

64 We overexpressed *Sctuf3*, where an overexpressed plasmid-borne copy of *Sctuf3* did confer
65 GE2270A resistance to *S. coelicolor*. To test this, the *Sctuf3* was PCR amplified using *S. coelicolor*
66 M145 (M1146 parental strain) genomic DNA as template, followed by cloning into pCR-Blunt to give
67 pBlunttuf3, which was confirmed by sequencing. Three different expression constructs of *Sctuf3* were
68 constructed:

- 69 • pIJ10257Atuf3: here *Sctuf3* is placed under the control of the strong and constitutive *ermE**
70 promoter in an integrative vector that uses phage BT1 integrase.
- 71 • pAV11bAtuf3: here *Sctuf3* is placed under the control of the aTc-inducible *tcp830* promoter
72 in an integrative vector that uses phage BT1 integrase.
- 73 • pIJ8781tuf3: this is a self-replicative construct where *Sctuf3* is placed under the control of the
74 thiostrepton-inducible *tipA* promoter in a multiple copy plasmid.

75 Each of the constructs were introduced into M1146 by intergeneric conjugation generating the
76 corresponding M1146::pIJ10257ASctuf3, M1146::pAV11bASctuf3 and M1146 (pIJ8781ASctuf3)
77 strains. A negative control strain, M1146::pIJ10257A, was also created during the same procedure.
78 Two independent clones per genotype were tested by agar diffusion bioassays using *Bacillus subtilis*
79 168 as indicator strain; when required, inducers, i.e., aTc (for pAV11b) and thiostrepton (for pIJ8781)
80 were added to the medium. None of the strains carrying an ectopic integrated copy of *Sctuf3* displayed
81 any increase in resistance either relative to their empty vector strain (M1146::pIJ10257tuf3, **Fig. S4A**)
82 or in the absence of induction (M1146::pAV11btuf3, **Fig. S4B**). However, the plasmid-borne version
83 of *Sctuf3* (M1146::pIJ8781tuf3), induced with thiostrepton, did lead to a resistant phenotype showing
84 at least 4x fold resistance relative to untransformed M1146 (**Fig. S4D**), although the growth of the
85 plasmid-bearing strains was severely affected (**Fig. S4C**).



86

87 **Figure S4. GE2270A tolerance of refactored strains expressing *Sctuf3* by agar diffusion assays with *B.***

88 ***subtilis* as indicator strain and by using known amounts of GE2270A. A) M1146::pIJ10257Atuf3 (two**

89 clones and M1146::pIJ10257A as control strain) B) M:::pAV11bAtuf3 (two clones uninduced and induced

90 conditions) and C) M1146 (pIJ8781tuf3) (two clones and parental strain as control). D) Liquid culture assays

91 testing M1146 (pIJ8781tuf3) in a 96-well plate by using known concentrations of GE2270A.

92 To investigate the phenotype of M1146 (pIJ8781tuf3) further, we performed liquid bioassays (in this

93 case, growth needed to be incubated for 72h instead of 48h, due to the slower growth rate of the

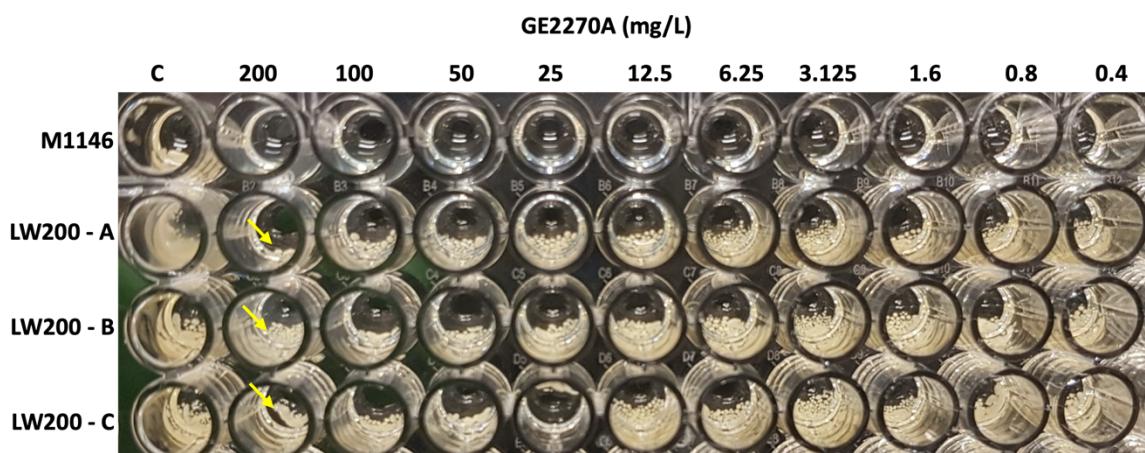
94 strains). Both clones of M1146 (pIJ8781 $tuf3$) showed at least 4 fold increased resistance to GE2270A
95 when the expression of *Sctuf3* was induced with thiostrepton, being able to grow even at 20 mg/L of
96 GE2270A, which was the highest concentration tested (Fig. S4D). Amongst all the different
97 recombinant strains generated in order to increase their tolerance to GE2270A, only
98 M1146::pIJ8781 $tuf3$ showed positive results, displaying an increased MIC of at least 4 fold in
99 comparison to the parental strain. This is consistent with the hypothesis that GE2270A-resistant forms
100 of EF-Tu need to be very highly expressed in order to functionally replace the house-keeping EF-Tu1.
101 However, this is not an optimal strain, because of its slow growth and its dependence on induction
102 (thiostrepton). This prompted us to seek other ways for improving intrinsic resistance, including the
103 isolation of spontaneous GE2270A-resistant mutants.

104

105 **Isolation of *S. coelicolor* M1146 spontaneous mutants resistant to GE2270A**

106 *Streptomyces coelicor* M1146 was repeatedly subcultured in media containing increasing sublethal
107 concentrations of GE2270A.

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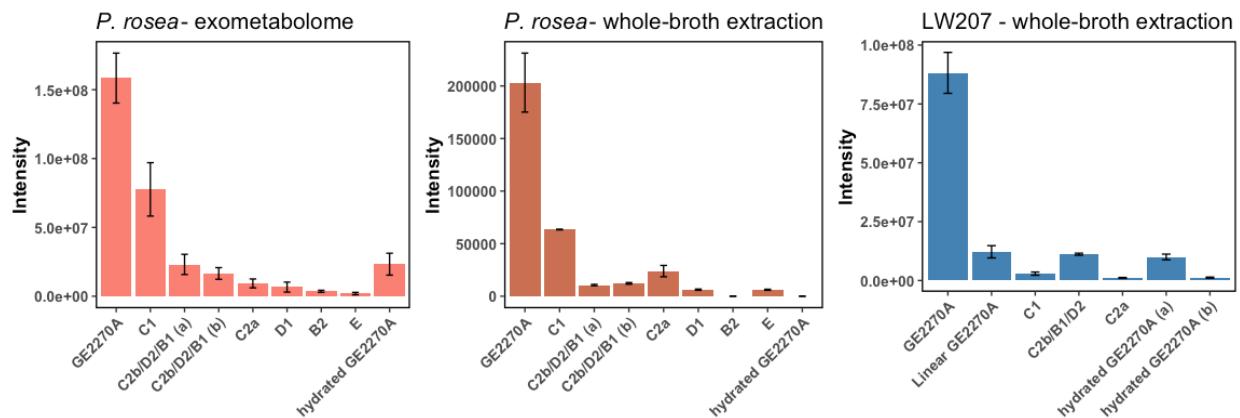


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110 **Figure S5. GE2270A tolerance of spontaneous mutant strain LW200.** Liquid bioassays of three biological
111 replicates of strain LW200 (A–C) and the parental strain M1146. Strains were grown in a 96-well microtitre plate
112 in the presence of a wide range of GE2270A concentrations.

113 **Comparison of relative levels of GE2270 congeners produced by different strains**

114 Using the previously published untargeted metabolomics data for *Planobispora rosea*⁵ and untargeted
115 metabolomics data acquired in this study, we compared the relative levels of congeners produced by
116 the natural producer and the LW207 strain.

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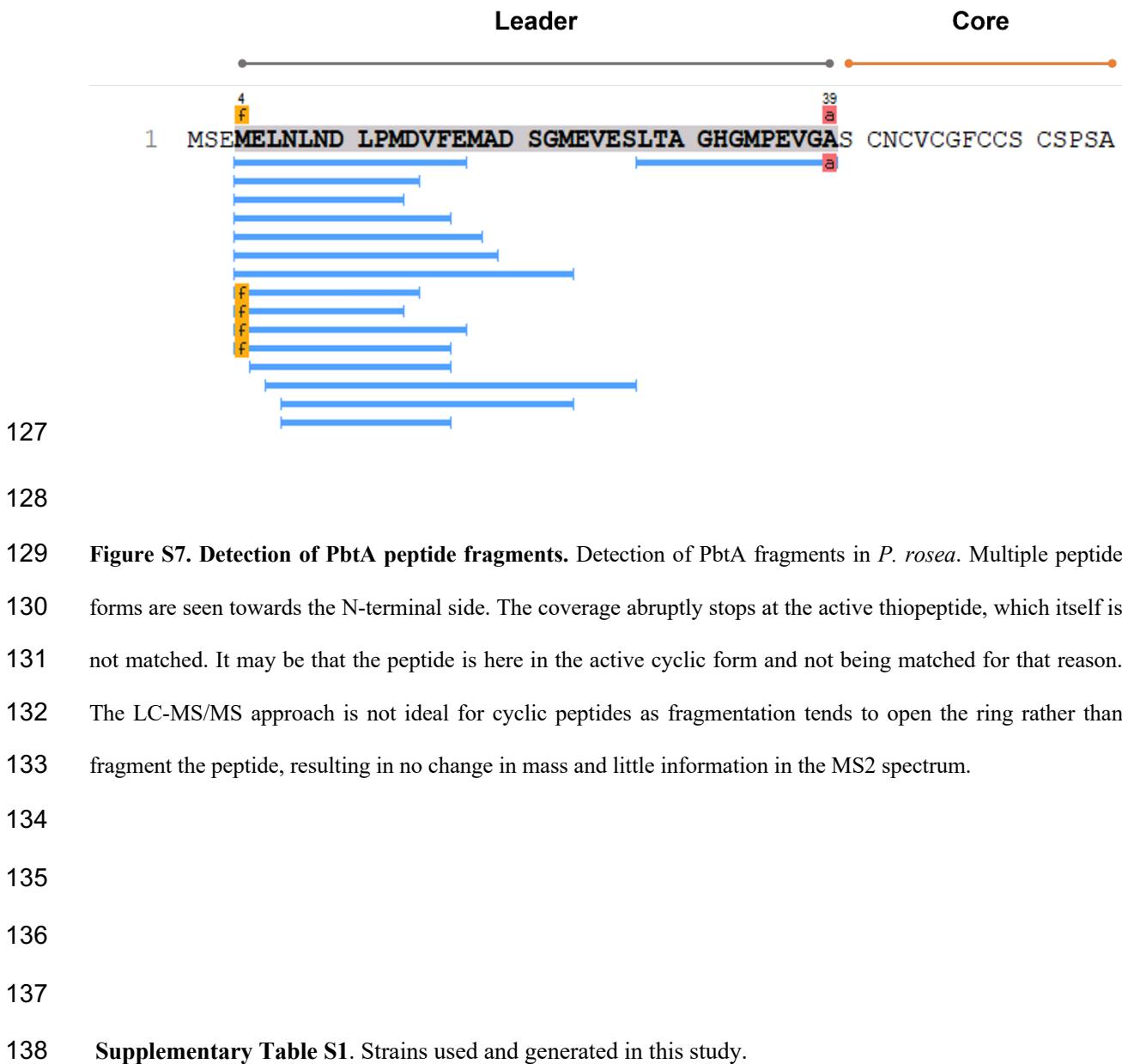
119 **Figure S6. Comparison of the GE2270 congeners produced by *P. rosea* and LW207.** The figure shows the
120 relative intensities associated with the culture of the two different strains at the latest timepoint, when the main
121 congener concentration (GE2270A) is the highest. The linear congener is only detected in the LW207 strain.

122

123

124 **Identification of true translational start site of PbtA through proteomics**

125 The proteomics analysis allowed the detection of the leader peptide as well as the identification of the
126 true translational start site of PbtA.



Strain	Genotype/characteristic	Reference or source
<i>E. coli</i>		
ET12567/pUB30 7	Conjugation donor	MacNeil <i>et al.</i> , 1992 ⁶ Bennet <i>et al.</i> , 1977 ⁷
ET12567/pUZ80 02	Conjugation donor	MacNeil <i>et al.</i> , 1992 ⁶

<i>E. coli</i> NEB5α	<i>fhuA2Δ(argF-lacZ)UI69</i> <i>phoA</i> <i>glnV44</i> <i>Φ80Δ(lacZ)M15</i> <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi-1</i> <i>hsdR17</i>	NEB
<i>S. coelicolor</i>		
M1146	<i>Δact Δred Δcpk Δcda</i>	Gomez-Escribano <i>et al.</i> , 2011 ⁸
LW200	M1146, spontaneous mutant tolerant GE2270A	This study
LW204	LW200::pbtCK02	This study
LW205	LW200::pbtCK02::pES118	This study
LW207	LW200::pbtCK02::pES118:: pES94*	This study
LW290	LW200::pTE1710	This study
LW291	LW200::pTE1711	This study
LW292	LW204::pTE1719	This study
LW293	LW200::pTE1712	This study
LW294	LW200::pTE1713	This study
LW295	Derivative of LW204; P _{ermE1} -pbtRG1B1O, P _{A9-} pbtXM1M2, P ₂₁ -pbtABCDEFGHM3M4	This study
LW296	LW204::pTE1720	This study
LW298	LW204::pTE1721	This study
<i>N. gerenzanensis</i>		
Nono-2F7	Nonomuraea::2F7	Flinspach <i>et al.</i> , 2014 ¹
Nono-cos3	Nonomuraea::SuperCos3	Flinspach <i>et al.</i> , 2014 ¹
Nono-Δ12	Nono::pTE1710	This work
Nono-Δ12pbtG1	Nono::pTE1711	This work

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143 **Supplementary Table S2.** Oligonucleotide primers used in this study.

Oligonucleotide primer name	Sequence (5' to 3')
3163_S4-pRT-f	tagcgggcagggagcggttgtggctggacaatcggtgcggtaggatc cagcggtatctaaggaggcaacaagtgcgtacaccggacc
3163_S4-pRT-r	acagctatgacatgattacgaattcgattcagcggagtagaaactccac
3163-UNS6-SspI-f	cgcgtccacctaagaatattaatacttacggcacatactgtaaggaggcaa caagtg
3163pbtAf-pRT-r	cacgggatcgcgaagactgttcagcggagtagaaactcca
A9_pbt02X-ps-r	gtatgtaccgttagagtattcttaggtggcagcgaacgagcagccccgtaga aaaga
A9_pbt02X-1.2-f	caagacgctggcttgacattccgtactgaactactcgacgctcagtgaaac gaaa
A9-pbt02X-fF-f	ctcgttcgctgccacctaagaataacttacggcacataccggctggcg gctgctc
A9_pbt02X-fF-r	ccctttatccttagtcaagtcaagcatgaggaagccggaaaaccgtattct cgactt
A9_pbt02X-fR-f	ccggcggcttcctcatgttgactttaggataaaggaaatcagcgg agaaaaga
A9_pbt02X-fR-r	cgagtagttcagtagcggaaatgtcagagccagcgtttggatgcggcag gccgtcg
A9_pbt02X-ps-f	gggatacggcgaatcagcggcagaaagagtttagagctagaaatagca

A9_pbt02X-2.1-r	agctctaaaactttctgctcgctgattcggcgatcccttcagatactcgac taa
E394	cgcaggctctgtacaggaaacagactatgac
E395	gtccggctctggccaatacgactcaactataggttcc
ermEpbt02R-fF-f	ctcgittcgctgccacctaagaatacttacggcacataccggaccggccactc gggtta
ermEpbt02R-fF-r	gctggatcctaccaaccggcacgattgtccagcccacaacataaataatcatcc cggtga
ermEpbt02R-fR-f	gggctggacaatctgccccgggttaggatccagcggtaacaaagtgcgtaa atgtccac
ermEpbt02R-fR-r	cgagtagttcagtagcggaaatgtcagagccagcgttgcacggcgagcgaa ccgagcgc
ermE-pbt02R-ps-f	aaaggggatacgctgacgcattcgatgatcggttttagagctagaaatagca
ermE-pbt02R-2.1-r	ctaaaaccacgatcatcgaaatcgctcagcgatcccttcagatact
p21-pbt02A-fF-f	ctcgittcgctgccacctaagaatacttacggcacataccatgtcgaggcg cacaag
p21_pbt02A-ps-f	tagtgcgagtatctgaaaggggatacgccgggcctccggatccgttt gagctagaaatagca
p21_pbt02-2.1-r	cggtatcccttcagatact
p21_pbt02A-fR-r	cgagtagttcagtagcggaaatgtcagagccagcgttgcggagacgttccg ccacggc
pbtAf3163-pRT-f	tggagttctactcccgctgaacagtcgttgcgtatccgtg

pbtAtr-pRT-f	ttggccttgcgaaatcggttaggttaggctaagttgtggctggacaatcggtccggtt ggtaggatccagcggtaacagtctcgatcccgtgt
pbtAtr-pRT-f	acagctatgacatgattacgaattcgatctcggtggccttctccacacg
pbtD-pRT3163-f	gctatgactcggtcgctgccaccataagaatatgacctggcgacgcgttgacg
pbtD-pRT3163-r	cttagcagtatgtgaccgttagagtattaaattcacatgccacccatggcgatctcg atggc
pbtG1-ermEp1-pSd1-f	agtgcgtggtcaccggggctgagtatacagttgtggctggacaatcggtcc ggttggtaggatccagcgggtaccgtggcgccggcat
pbtG1-pSd1-r	cacaggaaacagctatgacatgattacgtcgatccccattacgggaacggg tgggggt
pbtR-ermEp1-pES120-f	ttggccttgcgaaatcggttagtt
pbtR-UNS6-SspI-r	gaccgttagagtattaatattcttaggtggcagcgaacgagtcatagcaccatcg ggcgct
pbtR-O_ermEp1-pSd12-f	tgcgtggtcaccggggctgagtatacagttgtggctggacaatcggtccgg ttggtaggatccagcggtaacaaagtgcgtaatgtc
pbtR-O-pSd12-r	cacaggaaacagctatgacatgattacgtcgatccccattacctctgaatcg gattcc
pbtR_L92P_1.r	aagggtccggtaacgggcgacgtggca
pbtR_L92P_2.f	tgcacgtcgccgttacaccggaccctt
pbtR_L92P-f3	gaccggaaacgtgaccgtggccacttcg
pbtR_L92P-r2	cgaagtccggcacgggtacgttcgggtc

pCRM_2-1-f	gctgggagttcgtagacggaaacaaacgcagaatccaaggccatgcgctcat caagaa
pCRM_1-2-r	gcttggattctcggttgttccgtctacgaactcccagcggacgtgcttggca atca
pRT-overlap-r	ttagcctaactaacgattca
pSETpb1_frg1-v-f	tgcgaagcttggctgcaggcgactctagaggttgtggctggacaatcg ccggtgtggtaggatccagcggtaacaaagtgcgtaatgtccacctg
pSETpb1_frg1-r	cccggtcgccgagccacttcccgatt
pSETpb1_frg2-f	aactggagaagtggctgccgaccgg
pSETpb1_frg2-r	aaaaccgtattctcgactttcgctt
pSETpb1_frg3-f	aagagcgaaaagtgcgagaatacggtttccggggcttcatgcgtactt gactaggataaagggaatcagcgagcagaaagact
pSETpb1_frg3-r	ttgctcatcattaccatactaggacgtgttagagccgcacaaccgtggcatc cgccga
pSETpb1_frg4-f	tgtgcgggctctaacacgtcctagtatggtaggatgagcaaacagtctcg tcccg
pSETpb1_frg5-r	gctgccgatcatgaattccccgaccgcg
pSETpb1_frg6-f	cgcggtcgggaattcatgatcggcagc
pSETpb1_frg7-v-r	aggaaacagctatgacatgattacgtcgatccccatattctgaacttagtg ata ctcagccccgggtgac
pSpbt(cr)-frg1-f	ggccagtgccaagcttggctgcaggcgactctagagcggatggcttatga aggattgtctcacttaggatagagcacgaggacgcgtgtcgaaacg

pSpbt(c)n-F1-r	gaagatcctagccctcacattgatctgacagccctataaaaaaaaggctcaa aaggagccttaattcacggccccacctcgat
pSpbt(c)n-F2-f	tagaggctgtcagatcaatgtgagggctaggatctcaacactccgcctgcga gggggag
pSpbt(c)n-F2-r	ctggatcctaccaaccggcacgattgtccagcccacaactcagtggcatgact gcctcc
pSpbt(cr)-frg3-f	ggctggacaatcgtgccggttggtaggatccagcggtaacagtctcgcgt cccggt
pSpbt(c)n-F3-r	ttcacacagaaacagctatgacatgattcgaattcgattcgatccccaatattc tgaacttagtatcgctcgtggcttctccacacg

144

145 **Supplementary Table S3.** Plasmids used and generated in this study.

Plasmid	Characteristic	Reference or source
pbtCK02	<i>pbt</i> cluster, Hyg ^R , EF-Tu under control of <i>ermE</i> *p	Flinspach <i>et al.</i> , 2014 ¹
pCM4.4	<i>oriT-traJ</i> , <i>ermE</i> *p- <i>cas9</i>	Ye <i>et al.</i> , 2020 ⁹
pES90	<i>pbtR</i> under control of <i>kasO</i> *p	This study
pES94	<i>pbtR</i> under control of <i>kasO</i> *p	This study
pES103	<i>pbtA</i> under control of SP44p	This study
pES118	<i>pbtA</i> under control of <i>actII-orf4-actI</i> p	This study
pES120	<i>pbtR</i> under control of <i>ermEp1</i>	This study

pHG5	Contains <i>actII-orf4-actIp</i>	Gao and Smith, 2021 ¹⁰
pRT801	Integrative plasmid containing the BT1 integrase	Gregory <i>et al.</i> , 2003 ¹¹
pRTpbtA _{full}	<i>pbtA</i> under control of <i>ermEp1</i>	This study
pSET152	<i>oriT-traJ</i>	Blin <i>et al.</i> , 2016 ¹²
pTE1707	Insertion of <i>2lp</i> upstream of <i>pbtA</i>	This study
pTE1708	Insertion of <i>ermEp1</i> upstream of <i>pbtR</i>	This study
pTE1709	Insertion of <i>A9p</i> upstream of <i>pbtX</i>	This study
pTE1710	<i>pbt</i> cluster lacking <i>pbtRG1B1O</i> ; <i>ermEp1</i> and <i>A9p</i> upstream of <i>pbtX</i> , <i>2lp</i> upstream of <i>pbtA</i>	This study
pTE1711	<i>pbt</i> cluster lacking <i>pbtRB1O</i> ; <i>ermEp1</i> and <i>A9p</i> upstream of <i>pbtX</i> , <i>2lp</i> upstream of <i>pbtA</i> , <i>pbtG1</i> under control of <i>ermEp1</i>	This study
pTE1712	Expression of the <i>pbt</i> core cluster	This study
pTE1713	Expression of the refactored <i>pbt</i> cluster	This study

pTE1718	Expression of <i>3163S4</i> from <i>P. rosea</i>	This study
pTE1719	Expression of the full-length <i>pbtA</i> transcript and <i>3163S4</i> from <i>P. rosea</i>	This study
pTE1720	Expression of the full-length <i>pbtA</i> transcript, <i>pbtR</i> , and <i>3163S4</i> from <i>P. rosea</i>	This study
pTE1721	Expression of the full-length <i>pbtA</i> transcript, <i>pbtR</i> , <i>pbtD</i> , and <i>3163S4</i> from <i>P. rosea</i>	This study

146

147 **Supplementary Table S4.** Inclusion list containing the precursor ions selected for fragmentation as
 148 described in the ‘LC-MS metabolomics data acquisition’ section.

Name	[M+H] ⁺	[M+2H] ²⁺
Peak1	1297.21878	649.113027
Peak2	1305.24772	653.127498
Peak3	1345.29046	673.148867
Peak4	1327.27955	664.143413
Peak5	1292.26345	646.635361
Peak6	1091.15258	546.079929
Peak7	1276.22131	638.614295
Peak8	1108.17945	554.593361
Peak9	1433.3053	717.156286
Peak10	1362.26872	681.638

Peak11	1178.18451	589.595895
Peak12	1345.29011	673.148694
GE2270A	1290.26533	645.636305
Congener D1	1232.22347	616.615373
Linear Congener D2	1286.25516	643.63122
Linear Congener C1	1290.19256	645.599919
Congener E	1262.23403	631.620655
Congener C1	1246.23912	623.623198
Linear Congener E/D1	1316.26573	658.636502
Congener B1/C2b/D2	1276.24968	638.62848
Congener B2	1260.25477	630.631023
Linear CongenerB2	1314.28646	657.64687
Linear Congener B1/C2b	1330.28138	665.644327
Congener C2a	1306.26025	653.633762
Linear GE2270A	1344.29703	672.652152
Linear Congener C2a	1360.29194	680.649609
Linear GE2270A 2	1345.30485	673.156065
Core peptide	1569.48433	785.245805

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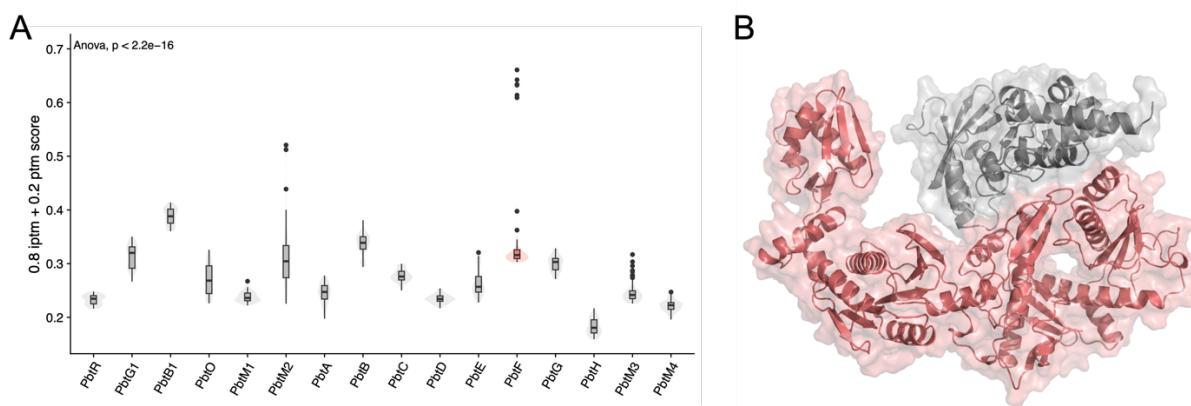
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156 **AlphaPullDown analysis of PbtX co-folding with the other members of the GE2270A BGC**

157

158 **Figure S8. AlphaPullDown analysis of PbtX co-folding with the other members of the GE2270A BGC. A)**
 159 The observed distribution of the $0.8 \text{ iPTM} + 0.2 \text{ PTM}$ scores all 50 models per each possible combination between
 160 PbtX and the other members of the GE2270A BGC. The protein that had the highest scored binding pose available
 161 (PbtF) is highlighted in red. B) The best ranked pose for PbtF (red) and PbtX (grey) which had the top 0.2 PTM
 162 $+ 0.8 \text{ iPTM}$ score across all models (0.66), and the second highest PI score¹³ across all models (2.33).

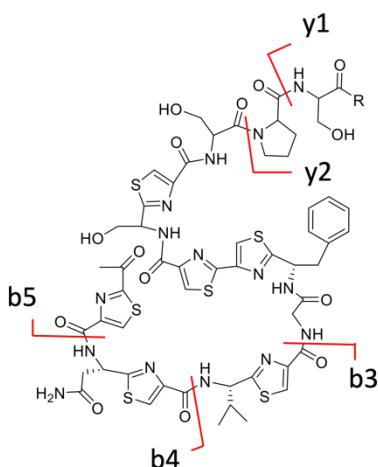
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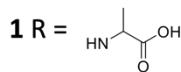
165 **Identification of previously unreported compounds**

166 Among the ten features represented in the similarity network (**Fig 3C** in the paper), two previously unreported
 167 compounds are shared by Nono-Δ12, Nono-Δ12G1, and Streptomyces LW291. These compounds have molecular
 168 formulas C58H64N16O16S6 (1, calculated 1432.3010, found 1432.2994) and C55H59N15O15S6 (2, calculated
 169 1361.2639, found 1361.2628), respectively. Both compounds appear to be linear as they share the fragment b3
 170 (**Fig S4**) with m/z 533.0736, which corresponds to the cleavage of the amide bond between alanine 7 and thiazole
 171 D (ThzD). This fragment lacks decorations on thiazole D, thiazole E (ThzE), and asparagine (Asn), and retains
 172 the N-terminal methylketone appendage on thiazole F (ThzF), as reported by Tocchetti *et al.* (2013)¹⁴. The primary
 173 distinction between compounds 1 and 2 lies in the presence of an alanine residue at the C-terminal position.
 174 Specifically, compound 1 contains serine and alanine following the C-terminal proline, as seen in the parental
 175 compound GE2270A, whereas compound 2 contains only serine. This difference is supported by the presence of
 176 fragment ions y2 at m/z 274.1397 for compound 1 and m/z 203.1026 for compound 2 (**Fig S4**). Additionally, the
 177 mass spectrometry data indicate rather than the absence of decorations on the thiazoles, also the absence of the

178 oxazoline ring and of the hydroxylation on the beta carbon of the phenylalanine residue.



	1		2	
	found	calculated	found	calculated
[M+2H] ²⁺	717.1569	717.1578	681.6386	681.6392
y1	177.0872	177.0870		
y2	274.1399	274.1397	203.1027	203.1026
b3	533.0745	533.0736	533.0733	533.0736
b4			351.0216	351.0222
b5			153.9960	153.9963



179

180 **Figure S9.** Identification of two previously unreported compounds shared by Nono-Δ12, Nono-Δ12G1, and
181 LW291.

182

183 Supplementary data

184 All supplementary data can be downloaded from here

185 https://www.dropbox.com/scl/fi/re9rrke6ea1g11s94nn9u/supplementary_data.zip?rlkey=2kkrpacvy8qyusc9xmhbrvpmx&dl=0

187

188 Supplementary Methods

189 Plasmid construction

190 Plasmid pES90 was constructed by GenScript. The synthesised fragment with the sequence
191 GAAGACTACTAGTGTTCACATTGAAACCGTCTCTGCTTGACAACATGCTGTGCGGTGTT

192 GTAAAGTCGTGGCCAGGAGAATACGACAGTCTAAGTAAGGAGTGTCGAAGTGACGAGCT
193 CGCGCAGTAATGCCAACGAGTGGTCCGAATCATCGACGCATCTGCGGAGCTCTCCTCC
194 AGAGGGATATCGACGGTGACCGTCGAGGAGGTGCCAGCCATGCCGGTGTCTCCAAG
195 AGCAGCGTCTACCTGCACTGGAACACGAAGGACGACATCTTCTACGACGCTCTGACCG
196 CGAGTGCGCCGCGCTCGTGTGCGAGGCCGTCGACCGCGTCAGACGCAACCCGGCCGAAA
197 TCCTGGCTCACCGGATGGCGGCCAATCTGCTCCGGATCATCCTGGACAGGCCGCTGCTGC
198 GGGCGCTGCTGATGGGTGACCAGCGATTCTGGATCGCTCCGCCATGCGAAGTCGTCC
199 GCTCTCCGATCCGGACGGCGCAATTGACGAGCTGATTACCGATACTCTCAGCGCTG
200 CAGAAAAACCAACTCATCTGTCCCACATCGATCTGCGCATCACCCGGAAAGCGGTGTG
201 GGAAATGCTGCGCGCATGATTTCCTGGCGGGACGGAGGCCGTTGGCAAGCCGCGCT
202 CCGCCGAGCTGGCGCAGGTATGACGGTTACCGTGCAGCGGGCGTTGAGGCCAGGGC
203 GTACCGGGGATCGACCGGATCACCGCCGCCGGCGAGGTGTTGAGGCAGTCGACGA
204 ACTCATTCCGACCGCGGAGAACCTGGACTTCGAGCGCCCGATGGTGTATGAACCTCATC
205 TGGATTGTTCAGAACGCTCGGTTGCCGCCGGCGTTTTAGGATCCAGTCTTC was
206 cloned into pUC57Kan by GenScript.

207 Plasmid pES94 was constructed by restriction enzyme-based cloning. The part *kasOp*-pblR-t0* was
208 excised from pES90 using BpiI and subsequently ligated into SpeI/BamHI-digested pRT801.

209 Plasmid pES103 was constructed by GenScript. The synthetised fragment with the sequence
210 ATTCCGGGGATCCGTCGACCTGTTCACATTGAAACCGTCTCTGCTTGACAACATGCTGT
211 GCGGTGTTGAAAGTCTGGTAGGTACCAATACGACTCACTATAGGTTCTGCTAAGGA
212 GGCAACAAGATGAGCGAGATGGAGTTAACCTAACGACCTGCCATGGACGTCTCGA
213 GATGGCCGACAGCGGCATGGAAGTTGAGTCACTCACCGCGGGACACGGGATGCCGAGG
214 TCGGTGCCTCGTGCACACTGCGTGTGCGGCTTCTGCTGTTCTGCAGCCCGTCCGCGTGAC
215 CGTCCAGCTCGACCAGAAAAAAAAAGCGCCGCAACTGCGGCGCTTTTTTCGAAG
216 CAGCTCCAGCCTACA was cloned into pUC57Kan by GenScript.

217 Plasmid pES118 was constructed by restriction enzyme-based cloning. The part RBS-*pbtA*-TT_{*sbiB*} was
218 amplified by PCR from pES103 using primers E395 and E394. The PCR product was digested using
219 BsaI and ligated into NcoI/Acc65I-digested pHG5.

220 Plasmid pES120 was assembled by restriction enzyme-based cloning. The gBlock with the sequence
221 GATGCAACTGGTCTCACTAGTACTGTTGTGGGCTGGACAATCGTGCCGGTTGGTAGGATC
222 CAGCGGGTACCAATACGACTCACTATAGTTCTGCTAAGGAGGCAACAAAATGACGAG
223 CTCGCGCAGTAATGCCAACGAGTGGTCCGAATCATCGACGCATCTGCGGAGCTCTCCT
224 CCAGAGGGATATCGACGGGTGACCGTCGAGGAGGTCGCCAGCCATGCCGGTGTCTCCA
225 AGAGCAGCGTCTACCTGCACTGGAACACGAAGGACGACATCTTCTACGACGCTCTGAC
226 CGCGAGTGC GCCCGCGCTCGTGTGCGAGGCCGTCACCGCGTCAGACGCAACCCGGCCGA
227 AATCCTGGCTCACCGATGGCGGCCAATCTGCTCCGGATCATCCTGGACAGGCCGCTGCT
228 GCGGGCGCTGCTGATGGGTGACCAGGCGATTCTGGATCGCTCCGCCATCGAAGTCGT
229 CCGCTCTCCGATCCCGAACGGCGGCCAATTGACGAGCTGATTACCGATATCTCTAGCGC
230 TGCAGAAAAACCAACTCATCTGTCGGACATCGATCTGCGCATCACCGAAAGCGGTG
231 TGGGAAATGCTGCGCGCATGATTTCCTCGCGGGACGGAGGCCGTTGGCAAGCCGCG
232 CTCCGCCGAGCTGGCGCAGGTATGACGGTTACCGTGC GGCGGGCGTTCGAGGCCAGGG
233 GCGTACCGGGATCGACCGGATCACGCCCGCGGCCGAGGTGTTCGAGGCCGTTCGAC
234 GAACTCATTCCGACCGCGGAGAACCTGGACTTCGAGCGCCGATGGTGTATGAACCTCC
235 ATCTGGATTGTTTCAGAACGCTCGGTTGCCGCCGGCGTTTTAGGATCCGGCCGGAA
236 GACCATGCGTCAG, synthesised by GenScript, was digested with BsaI and combined with
237 SpeI/NotI-digested pRT801.

238 Cosmid pTE1710 was constructed by HiFi DNA Assembly of five parts. The first part, comprising
239 *pbtRBIG1O*, was amplified by PCR from pbtCK02 using primers pSETpbt1_frg1-v-f and
240 pSETpbt1_frg2-r. The second part, comprising *pbtXMIM2*, was amplified by PCR from pbtCK02
241 using primers pSETpbt1_frg3-f and pSETpbt1_frg3-r. The third part, comprising *pbtABCDE*, was
242 amplified by PCR from pbtCK02 using primers pSETpbt1_frg4-f and pSETpbt1_frg5-r. The fourth

243 part, comprising *pbtFGHM3M4*, was amplified by PCR from pbtCK02 using primers pSETpbt1_frg6-
244 f and pSETpbt1_frg7-v-r. The PCR products were combined with BamHI/EcoRV-digested pSET152.

245 Cosmid pTE1711 was constructed by HiFi DNA Assembly of two parts. The first part, comprising
246 *pbtG1* under control of *ermEp1*, was amplified by PCR from pbtCK02 using primers pbtG1-ermEp1-
247 pSd1-f and pbtG1-pSd1-r. The PCR product was combined with SpeI/SspI-digested pTE1710.

248 Cosmid pTE1712 was constructed by HiFi DNA Assembly of five parts. The first part, comprising
249 *pbtG1B1*, was amplified by PCR from pbtCK02 using primers pSpbt(cr)-frg1-f and pSpbt(c)n-F1-r.
250 The second part, comprising *pbtBCDE*, was amplified by PCR from pbtCK02 using primers
251 pSpbt(c)n-F2-f and pSETpbt1_frg5-r. The third part, comprising *pbtFG* was amplified by PCR from
252 pbtCK02 using primers pSETpbt1_frg6-f and pSpbt(c)n-F2-r. The fourth part, comprising *pbtA*, was
253 amplified by PCR from pbtCK02 using primers pSpbt(cr)-frg3-f and pSpbt(c)n-F3-r. The PCR
254 products were combined with BamHI/EcoRV-digested pSET152.

255 Cosmid pTE1713 was constructed by HiFi DNA Assembly of three parts. The first part was amplified
256 by PCR from pbtCK02 using primers pbtR-O_ermEp1-pSd12-f and pSETpbt1_frg1-r. The second
257 part was amplified by PCR from pbtCK02 using primers pSETpbt1_frg2-f and pbtR-O-pSd12-r. The
258 PCR products were combined with SpeI/SspI-digested pTE1710.

259 Cosmid pTE1718 was constructed by HiFi DNA Assembly of two parts. The first part was amplified
260 by PCR from *P. rosea* genomic DNA using primers 3163_S4-pRT-f and 3163_S4-pRT-r. The PCR
261 product was combined with BamHI/EcoRV-digested pRT801.

262 Cosmid pTE1719 was constructed by HiFi DNA Assembly of two parts. The first part was amplified
263 by PCR from pTE1718 using primers pbtR_L92P-f3 and 3163pbtAf-pRT-r. The second part was
264 amplified by PCR from pRTpbtA_{full} using primers pbtR_L92P-r2 and pbtAf3163-pRT-f.

265 Cosmid pTE1720 was constructed by HiFi DNA Assembly of three parts. The first part was amplified
266 by PCR from pES120 using primers pbtR-ermEp1-pES120-f and pbtR-UNS6-SspI-r. The second part
267 was amplified by PCR from pTE1719 using primers 3163-UNS6-SspI-f and pbtR_L92P_1.r. The
268 third part was amplified by PCR from pTE1719 using primers pRT-overlap-r/pbtR_L92P_2.f.

269 Cosmid pTE1721 was constructed by HiFi DNA Assembly of two parts. The first part, comprising
270 *pbtD*, was amplified by PCR from pbtCK02 using primers pbtD-pRT3163-f and pbtD-pRT3163-r.
271 The PCR product was combined with SspI-digested pTE1720.

272 Plasmid pRTpbtA_{full} was constructed by HiFi DNA Assembly of two parts. The first part was
273 amplified by PCR from pbtCK02 using primers pbtAtr-pRT-f and pbtAtr-pRT-r. The PCR product
274 was combined with EcoRV/SpeI-digested pRT801.

275 Plasmid pTE1707 was constructed by Hifi DNA assembly of five parts. The first part, comprising the
276 upstream homology region, was amplified by PCR from pbtCK02 using primers p21-pbt02A-fF-f and
277 pSETpbt1_frg3-r. The second part, comprising the downstream homology region, was amplified by
278 PCR from pbtCK02 using primers p21_pbt02A-fR-r and pSETpbt1_frg4-f. The third part, comprising
279 the protospacer, was amplified by PCR from pCM4.4 using primers p21_pbt02A-ps-f and
280 A9_pbt02X-ps-r. The fourth part, comprising the first part of the backbone, was amplified by PCR
281 from pCM4.4 using primers p21_pbt02-2.1-r and pCRM_2-1-f. The fifth part, comprising the second
282 part of the backbone, was amplified by PCR from pCM4.4 using primers pbt02X-1.2-f and pCRM_1-
283 2-r.

284 Plasmid pTE1708 was constructed by Hifi DNA assembly of five parts. The first part, comprising the
285 upstream homology region, was amplified by PCR from pbtCK02 using primers ermEpbt02R-fF-f
286 and ermEpbt02R-fF-r. The second part, comprising the downstream homology region, was amplified
287 by PCR from pbtCK02 using primers ermEpbt02R-fR-f and ermEpbt02R-fR-r. The third part,
288 comprising the protospacer, was amplified by PCR from pCM4.4 using primers ermE-pbt02R-ps-f
289 and A9_pbt02X-ps-r. The fourth part, comprising the first part of the backbone, was amplified by
290 PCR from pCM4.4 using primers ermE-pbt02R-2.1-r and pCRM_2-1-f. The fifth part, comprising the
291 second part of the backbone, was amplified by PCR from pCM4.4 using primers pbt02X-1.2-f and
292 pCRM_1-2-r.

293 Plasmid pTE1709 was constructed by Hifi DNA assembly of five parts. The first part, comprising the
294 upstream homology region, was amplified by PCR from pbtCK02 using primers A9-pbt02X-fF-f and
295 A9_pbt02X-fF-r. The second part, comprising the downstream homology region, was amplified by

296 PCR from pbtCK02 using primers A9_pbt02X-fR-f and A9_pbt02X-fR-r. The third part, comprising
297 the protospacer, was amplified by PCR from pCM4.4 using primers A9_pbt02X-ps-f and A9_pbt02X-
298 ps-r. The fourth part, comprising the first part of the backbone, was amplified by PCR from pCM4.4
299 using primers A9_pbt02X-2.1-r and pCRM_2-1-f. The fifth part, comprising the second part of the
300 backbone, was amplified by PCR from pCM4.4 using primers A9_pbt02X-1.2-f and pCRM_1-2-r.
301

302 **References**

- 303
- 304 1. Flinspach, K., Kapitzke, C., Tocchetti, A., Sosio, M. & Apel, A. K. Heterologous
305 Expression of the Thiopeptide Antibiotic GE2270 from Planobispora rosea ATCC
306 53733 in *Streptomyces coelicolor* Requires Deletion of Ribosomal Genes from the
307 Expression Construct. *PLoS One* **9**, e90499 (2014).
 - 308 2. Cobb, R. E., Wang, Y. & Zhao, H. High-Efficiency Multiplex Genome Editing of
309 *Streptomyces* Species Using an Engineered CRISPR/Cas System. *ACS Synth Biol* **4**,
310 723–728 (2015).
 - 311 3. van Wezel, G. P., Takano, E., Vijgenboom, E., Bosch, L. & Bibb, M. J. The tuf3 gene
312 of *Streptomyces coelicolor* A3(2) encodes an inessential elongation factor Tu that is
313 apparently subject to positive stringent control. *Microbiology (N Y)* **141**, 2519–2528
314 (1995).
 - 315 4. Olsthoorn-Tieleman, L. N., Palstra, R.-J. T. S., van Wezel, G. P., Bibb, M. J. & Pleij,
316 C. W. A. Elongation Factor Tu3 (EF-Tu3) from the Kirromycin Producer *Streptomyces*
317 *ramocissimus* Is Resistant to Three Classes of EF-Tu-Specific Inhibitors. *J Bacteriol*
318 **189**, 3581–3590 (2007).
 - 319 5. Del Carratore, F. et al. Multi-omics Study of Planobispora rosea, Producer of the
320 Thiopeptide Antibiotic GE2270A. *mSystems* **6**, (2021).
 - 321 6. MacNeil, D. J. et al. Analysis of *Streptomyces avermitilis* genes required for
322 avermectin biosynthesis utilizing a novel integration vector. *Gene* **111**, 61–68 (1992).
 - 323 7. Bennett, P. M., Grinsted, J. & Richmond, M. H. Transposition of TnA does not
324 generate deletions. *Mol Gen Genet* **154**, 205–211 (1977).
 - 325 8. Gomez-Escribano, J. P. & Bibb, M. J. Engineering *Streptomyces coelicolor* for
326 heterologous expression of secondary metabolite gene clusters. *Microb Biotechnol* **4**,
327 207–215 (2011).
 - 328 9. Ye, S., Enghiad, B., Zhao, H. & Takano, E. Fine-tuning the regulation of Cas9
329 expression levels for efficient CRISPR-Cas9 mediated recombination in
330 *Streptomyces*. *J Ind Microbiol Biotechnol* **47**, 413–423 (2020).
 - 331 10. Gao, H. & Smith, M. C. M. Use of orthogonal serine integrases to multiplex plasmid
332 conjugation and integration from *E. coli* into *Streptomyces*. *Access Microbiol* **3**,
333 (2021).

- 334 11. Gregory, M. A., Till, R. & Smith, M. C. M. Integration Site for *Streptomyces* Phage
335 φBT1 and Development of Site-Specific Integrating Vectors. *J Bacteriol* **185**, 5320–
336 5323 (2003).
- 337 12. Blin, K., Pedersen, L. E., Weber, T. & Lee, S. Y. CRISPy-web: An online resource to
338 design sgRNAs for CRISPR applications. *Synth Syst Biotechnol* **1**, 118–121 (2016).
- 339 13. Malhotra, S., Joseph, A. P., Thiyyagalingam, J. & Topf, M. Assessment of protein–
340 protein interfaces in cryo-EM derived assemblies. *Nat Commun* **12**, 3399 (2021).
- 341 14. Tocchetti, A. *et al.* Capturing Linear Intermediates and C-Terminal Variants during
342 Maturation of the Thiopeptide GE2270. *Chem Biol* **20**, 1067–1077 (2013).
- 343