GRANT AGREEMENT NO 2019-1-PT01-KA203-061449
ERASMUS+ PROGRAMME – KA2 – STRATEGIC PARTNERSHIP

HYBRID
LAB NETWORK

CRISPR – Cas9 editing of *Streptomyces* genome

In vivo triggering of CAS9 system to switch off blue pigment production in *Streptomyces coelicolor.*

Protocol to inhibit actinorhodin production in *S. coelicolor* by inactivating *actIORF1* or *actVB* genes of the blue-pigmented actinorhodin biosynthetic pathway. This protocol is based on the procedures described in Tong *et al.* (2015) and Tong *et al.* (2020).

Biological Materials

- *Streptomyces coelicolor* M145 (ATCC BAA-471). (Biosafety Level 1)
- *Escherichia coli* DH5α. Thermo Fisher Scientific, cat. n° 18265017. These cells are used for cloning procedures. (Biosafety Level 1)
- *E. coli* ET12567/pUZ8002. Maintaining *E. coli* ET12567 cells requires both chloramphenicol 25 µg/mL and kanamycin 50 µg/mL, because *E. coli* ET12567 has a chloramphenicol-resistance marker and the carrier plasmid pUZ8002 has a kanamycin-resistance marker. Not commercially available. *E. coli* ET12567 is a methylation defective strain and the pUZ8002 plasmid contains *tra* gene, which can transfer the protein Tra to facilitate DNA transfer. (Biosafety Level 1)

Reagents

MS agar plates: Make MS (Mannitol Soya Flour) agar plates by mixing well (with a magnetic stirrer) 20 g/L D-mannitol, 20 g/L fat-reduced soy flour, and 20 g/L agar in normal tap water. Adjust the pH to 8 with 1 M NaOH before autoclaving. After autoclaving at 121 °C for 20 min, add pre-autoclaved $MgCl_2$ to a final concentration of 10 mM; then use a magnetic stirrer to mix until the temperature naturally lowers to aprox 50 °C for pouring into Petri dishes.

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ISP4 agar plates: Make ISP4 agar plates by dissolving premixed Difco ISP4 browth powder (BD, cat n° 277210) in deionized water. After autoclaving (121 °C, 20 min), naturally cool down the medium at room temperature. Before use, add antibiotics when needed.

TSA plates: Make TSA agar plates by dissolving 30 g/L of TSB (Tryptone Soy Broth) broth powder (Neogen, cat. n° LAB004) in deionized water and mixing 20 g/L agar. After autoclaving at 121°C for 15 min, naturally cool down the medium at room temperature, add antibiotics when needed and pour into Petri dishes**.**

LB broth: Make LB broth by dissolving premixed LB broth powder (*Sigma-Aldrich, cat. n° L3022-1kg*) in normal tap water and adjust the pH to 7.0 with 1 M NaOH before autoclaving. After autoclaving (121 °C, 20 min), naturally cool down the medium at room temperature. Before use, add antibiotics when needed.

2xYT: Make 2× YT broth by mixing 16 g/L of tryptone, 10 g/L of yeast extract, and 5 g/L of NaCl in normal tap water using a magnetic stirrer. Adjust the pH to 7.0 with 1 M NaOH before autoclaving (121 °C, 20 min).

Apramycin stock solution (50 mg/mL): Use an electronic scale to weigh 500 mg apramycin sulphate salt (*Sigma-Aldrich, cat. no. A2024-5G*) and dissolve it in 10 mL ddH2O. Filter the solution with a 0.2-µm filter in a laminar flow hood and then make aliquots in sterilized 1.5-mL Eppendorf tubes. The stock can be stored at −20 °C for up to 1 year.

Nalidixic acid stock solution (50 mg/mL): Use an electronic scale to weigh 500 mg nalidixic acid (*Sigma-Aldrich, cat. no. N8878-5G*) and dissolve it in 10 mL 0.1 M NaOH. Filter the solution with a 0.2-µm filter in a laminar flow hood and then make aliquots in sterilized 1.5-mL Eppendorf tubes. The stock can be stored at −20 °C for up to 1 year.

Theophylline stock solution (150 mM): Weigh 270 mg theophylline (Sigma-Aldrich, cat. no. T1633-50G) and dissolve it in 10 mL 0.1 M NaOH. Filter the solution with a 0.2-µm filter in a laminar flow hood.

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A. *Streptomyces coelicolor* spore suspension

PART 1

1. Plate *Streptomyces coelicolor* onto MS agar plates for sporulation. Inoculate 10 µL of a 10^8 cfu/mL spore suspension into MS plates using a sterilized inoculating loop (*Sigma-Aldrich, cat n° I8388-500EA*). Incubate for 5-6 d at 30°C or until plates are fully sporulated (a typical well-sporulated *S. coelicolor* plate presents a greyish colour).

1 WEEK INTERVAL

PART 2

2. Carefully pipet 10 mL of sterile 20% (w/v) glycerol + 0.025% (v/v) triton X-100 onto the surface of the spore lawn.
3. Use a sterilized inoculating 10 µL loop to gently scraping the surface of the culture and suspend the spores by pipetting up and down several times.
4. Filter the suspension through non-absorbent cotton wool, using a sterile syringe or a 5 mL tip with a cotton plug at the bottom.
5. Collect the spores by centrifugation at 5000 g for 5 min at room temperature. Discard the supernatant carefully, resuspend the spores in 0.5-1 mL 20% (w/v) glycerol + 0.025% (v/v) triton X-100. *S. coelicolor* spore suspension can be stored at -80 °C.
6. Determine the spore suspension concentration by measuring the absorbance at 600 nm (OD600 = 1 corresponds to 10^8 cfu/mL).

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B. sgRNA generation

1. Two CRISPR spacers, previously described (Tong et al., 2015), were selected targeting two genes from the actinorhodin biosynthetic pathway in *S. coelicolor*:

   ACT1 - GCGCGAGTATCTGCTGCTGT targeting the SCO5087/ *actIORF1* gene
   ACT5 - ATCTTCGAACTCCCTAGGCG targeting the SCO5092 gene

2. Design and order (standard desalting protocol) ssDNA oligonucleotides for protospacer integration into CRISPR plasmid (pCRISPR-TT). Oligonucleotides include a NcoI recognition site (bold) and the 20nt spacer sequence (underlined):

ACT1_F: 5' - CATG**CCATGG**GCGCGAGTATCTGCTGCTGTGTTTTAGAGCTAGAAATAGC - 3'
ACT5_F: 5' - CATG**CCATGG**ATCTTCGAACTCCCTAGGCGGTTTTAGAGCTAGAAATAGC - 3'

3. Resuspend the oligonucleotides in water at a 100 µM stock concentration. Dilute the oligonucleotides to a working concentration of 10 µM.

4. sgRNA generation for integration into pCRISPR-TT plasmid (Fig. 1) requires PCR. Carry out a PCR for each sgRNA using the pCRISPR-TT plasmid as template, the spacer containing primers designed in step B.2 as forward primer and the sgRNA_R primer as reverse primer. The sequence of the sgRNA_R primer is 5' - ACGCC**TACGTA**AAAAAAGCACCGACTCGGTGCC - 3' and it includes a SnaBI recognition site (bold). PCR setup and conditions are as follows:

Component	**Final Concentration**	**Volume (25 µL reaction)**
Forward Primer, 10 µM	100 nM	0.25 µL
Reverse primer - sgRNA_R, 10 µM	100 nM	0.25 µL
Template DNA	10 ng	variable
dNTP, 10 mM	200 µM	0.5 µL
5x Q5 reaction buffer	1x	5 µL
Q5 High-Fidelity DNA Polymerase (New England BioLabs, cat n° M0491)	0.02 U/µL	0.25 µL
Water	-	up to 25 µL

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PCR conditions: Denature 5'@95°C; 35x [denature 10''@95°C; anneal and extension 60''@72°C]; final extension 5'@72°C.

5. Analyse 5 µL of each PCR reaction on an agarose gel (1% (w/v)). A band of 122 bp is expected.

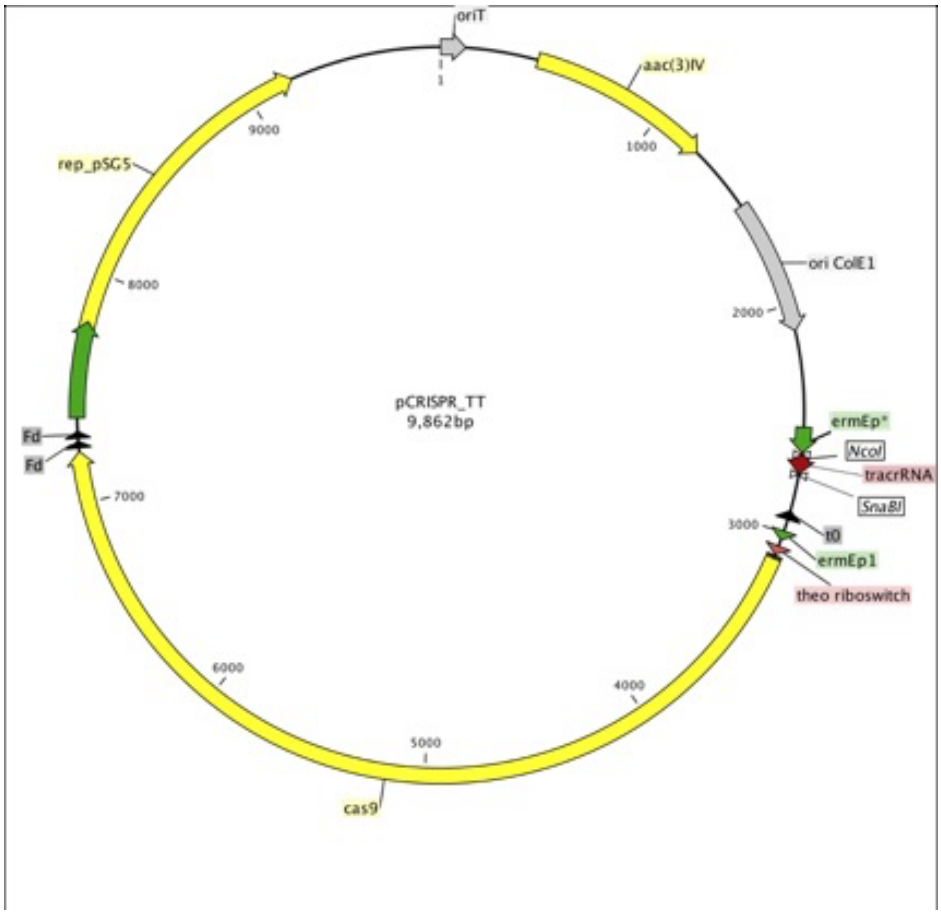

Figure 1 - Map of pCRISPR-TT map featuring a temperature sensitive pSG5 replicon, the cas9 gene controlled by the constitutive ermEp1 promoter with the theophylline riboswitch, the sgRNA cassette under the control of the ermE*p promoter and apramycin as the selection marker.

6. Purify the PCR fragments using a PCR clean-up kit according to the manufacturer's instructions. Determine the concentration of each fragment using a NanoDrop spectrophotometer.

C. Construction of CRISPR plasmid

C1. Assembling the CRISPR plasmid

1. Digest the sgRNA fragments generated in step B.6 using NcoI and SnaBI. Set up a 20 µL reaction and incubate for 2h at 37°C.

2. Linearize the pCRISPR-TT plasmid (aprox 800 ng) with NcoI and SnaBI in a 20 µL digestion reaction. Incubate at 37°C for 1h30. Add 1 µL FastAP thermosensitive alkaline phosphatase to the reaction and incubate for additional 10 min at 37°C. Confirm plasmid digestion by analyzing 5 µL of the reaction on an agarose gel.

3. Clean up the both digestion reactions from steps C.1 and C.3 with a clean-up kit according to the manufacturer's instructions.

4. To clone the sgRNA fragments into the linearized pCRISPR-TT plasmid prepare a 20 µL reaction mix as follows:

Component	**Final Concentration**	**Volume (20 µL reaction)**
Linearized plasmid from Step C.3	50 ng	variable
Purified sgRNA fragment from step C.3	200 ng	variable
10x T4 DNA ligase reaction buffer, 10x	1x	2 µL
T4 DNA ligase (Thermo Scientific, cat n° EL0011)	0.05 U/µL	0.2 µL
Water	-	up to 20 µL

5. Incubate the reaction O/N at 4°C.

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C2. Transformation of CRISPR plasmid into *E. coli*

6. Transfer 5 µL of the above reaction mix to 50 µL of in-house-made (or commercial) chemically competent *E. coli* DH5α using a heat-shock protocol as follows:

    a. thaw on ice a 50-µL aliquot of competent cells;

    b. mix with 5 µL of the reaction from step C.4

    c. Incubate on ice for 20 min, followed by a 90 s heat shock at 42°C, using a water bath. Incubate on ice for another 5 min.

    d. Add 200 µL SOC or LB medium to the tube and incubate at 37°C with shaking for 45-60 min.

    e. plate all the reaction on a selective LB plate supplemented with 50 µg/mL apramycin. Incubate the plate O/N at 37°C.

7. Screen the clones using an *E. coli* colony PCR as follows: use sterilized wooden toothpicks to partially pick 10-20 *E. coli* colonies (by touching the colony) and transfer to a 1,5 mL Eppendorf tube containing 50 µL of sterilized water. Homogenize the solution by vortexing for 30 s. Directly use 5 µL of the solution as template DNA for colony PCR with the following setup and conditions:

Component	**Final Concentration**	**Volume (20 µL reaction)**
Forward Primer - pUCR, 10 mM	100 nM	0.2 µL
Reverse primer - sgRNA_R, 10 mM	100 nM	0.2 µL
Template DNA	-	5 µL
dNTP, 10mM each	200 µM each	0.4 µL
5x GoTaq® reaction buffer	1x	4 µL
GoTaq DNA Polymerase (5U/µL) (Promega, cat n° M3001)	1.25 U	0.25 µL
Water	-	9.95 µL

PCR conditions: Denature 10'@95°C; 35x [denature 30''@95°C; anneal 30'' @55°C and extension 30''@72°C]; final extension 10'@72°C.

8. Analyse 10 µL of the above PCR reaction (add 2 µL of 6x DNA gel loading dye) on an agarose gel 2% (w/v) with 1xTAE buffer. Clones with correct integration of the sgRNA originate a band with an expected size of 271 bp (vs 251 bp of control reaction using pCRISPR-TT plasmid as template DNA).

9. Select 2-3 above obtained positive clones and prepare cultures by inoculating positive colonies into 5 mL of LB medium supplemented with 50 µg/mL apramycin. Incubate O/N with shaking (150 rpm) at 37°C.

10. Perform CRISPR plasmid isolation using a plasmid isolation kit and following the manufacturer's instructions (eg: *NucleoSpin Plasmid EasyPure Kit*). Confirm the identity of the obtaining plasmids by restriction mapping and Sanger sequencing. *E. coli* strains with the correct plasmids can be stored in 25% (v/v) glycerol at -80°C for long-term storage.

D. transfer of CRISPR plasmid into *S. coelicolor* by conjugation

1. Transfer 200 ng of the desired plasmids from Step C.10 into 50 µL of in-house-made electroporation competent *E. coli* ET12567/pUZ8002 cells, using an electroporation protocol. Instead of plating onto selective LB plates, transfer all the reaction to a 50-mL Falcon tube with 20 mL selective LB medium supplemented with 50 µg/mL apramycin, 25 µg/mL kanamycin, and 12.5 µg/mL chloramphenicol. Incubate the tubes overnight at 37 °C with 200 r.p.m. shaking.

2. The following morning, prepare the ET cultures harbouring the plasmids of interest, by washing twice using the same volume (20 mL) of antibiotic-free LB medium, and harvest the cells by centrifuging at 5,000g for 5 min at room temperature. Then resuspend the cell pellets in 2 mL antibiotic-free LB medium per 20-mL of culture.

3. Resuspend aprox 10^8 spores from the *S. coelicolor* spore suspension prepared in step A.5 in 500 µL of 2xYT.

4. Heat-shock the spore suspension for 10 min at 50 °C. The spore suspension is now ready for conjugation.

5. Mix 500 µL ET suspension from step D.2 with 200 µL spore suspension from step D.4 in a sterilized 1.5-mL Eppendorf tube by pipetting.

6. Plate 200 µL and 400 µL of the mixture from step D.5 onto different MS plates, air-dry them in a laminar flow hood for 5 min, and incubate the conjugation plates at 30 °C for ~24 h (overnight).

7. Overlay the conjugation plates with 1 mL sterilized H_2O containing 1 mg apramycin and 1 mg nalidixic acid. Thoroughly spread across the whole plate the 1 mL sterilized H_2O containing 1 mg apramycin and 1 mg nalidixic acid just by moving the plate. Air-dry the plates in a laminar flow hood for 15 min.

8. Incubate the plates until exconjugants can be picked with a sterilized wooden toothpick. Typically, it takes 5–7 d. Transfer the picked exconjugants to a fresh ISP4 plate supplemented with 50 µg/mL apramycin and 50 µg/mL nalidixic acid and incubate for 5–6 d at 30 °C.

9. Select 2-3 apramycin resistant exconjugants and prepare a spore suspension following the procedure in steps A.

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E. Induction of cas9 expression

1. Plate *S. coelicolor* exconjugant strains obtained in step D.9 onto TSA or/and ISP4 agar plates supplemented with 50 µg/mL apramycin and 5 mM theophylline for induction of *cas9* expression. Spread 100 µL of a 10^3 cfu/mL spore suspension into TSA or/and ISP4 plates using a sterilized inoculating spreader (eg. *Sarstedt, cat nº 86.1569.005*). Incubate for 5-6 d at 30ºC or until colony blue/red pigmentation is observed. Genetically edited strains do not display the blue pigmentation phenotype.

References

1. Rudolph, M. M., M. P. Vockenhuber and B. Suess (2013). Synthetic riboswitches for the conditional control of gene expression in *Streptomyces coelicolor*. *Microbiology* **159** (Pt 7): 1416-1422.
2. Tong, Y., P. Charusanti, L. Zhang, T. Weber and S. Y. Lee (2015). CRISPR-Cas9 based engineering of actinomycetal genomes. *ACS Synth Biol*.
3. Tong, Y., C. M. Whitford, K. Blin, T. S. Jorgensen, T. Weber and S. Y. Lee (2020). CRISPR-Cas9, CRISPRi and CRISPR-BEST-mediated genetic manipulation in *streptomycetes*. *Nat Protoc* **15** (8): 2470-2502.

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