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🌐 pYCR cloning strategy

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ABSTRACT

Introducing NLP14a in the genome of *B. mycoides*: In order to create plasmid pYCR-gamyNLP, the backbone of Pycr will be digested with the PCR product of gamy_Fw and gamy_Rv to produce the pYCR_gamy (CRISPR vector containing the gRNA). The NLP14a sequence will be ordered from Twist Biosciences And PCR-amplified with the primers NLP_Fw and NLP_Rv. The resulting PCR product, as well as the pYCR_gamy vector will be digested with Sfil. The digested mixtures will be ligated using T4 ligase.

Kill switch cloning: The suggested kill switch mechanism is based on Trp auxotrophic strains as well as a toxin-antitoxin mechanism. The toxin will be introduced in the genome using crispr in place of trpE. The antitoxin will be maintained as a cytoplasmic plasmid (pAD-YqcF) and expressed only in the presence of solanine. Unfortunately, to the best of our knowledge, no operator that binds solanine of *B. mycoides* has been described in literature. If it is provebn that no such operator is present, we suggest on oly keeping the tryptophan dependence strategy.

In order to create plasmid pYCR-gtrpEYqcG plasmid, the backbone of Pycr will be digested with Bsal and ligated with the annealing product of gtrpe_Fw and gtrpe_Rv to produce the pYCR_gtrp (CRISPR vector containing the gRNA). Synthetic dna for *yqcG* will be ordered from Twist Biosciences and PCR-amplified with the primers Yqcg_Fw and Yqcg_Rv. The resulting PCR product, as well as the pYCR_gtrpE vector will be digested with Sfil. The digested mixtures will be ligated using T4 ligase

Protocol was adapted after a paper of Yi et al., 2018

1. Yi, Y., Li, Z., Song, C. & Kuipers, O. P. Exploring plant-microbe interactions of the rhizobacteria *Bacillus subtilis* and *Bacillus mycoides* by use of the CRISPR-Cas9 system. *Environ. Microbiol.* 20, 4245–4260 (2018).

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Protocol status: Other Protocol has been developed based on literature, but hasn't been tested yet.

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PROTOCOL integer ID: 43671

Clone sgRNA sequence into pYCR

1 Design sgRNA spacer sequence (~20 nts) using "Benchling" and choose *B. mycoides* M2E_15 genome
Choose the gRNA with the highest on-target score and the lowest off-target score.

2 Order the 2 complementary oligos flanked by overhang containing BsaI restriction site

3 Anneal oligos by mixing the following components:

- 70 µL Nuclease Free water
- 10 µL Oligo DNA annealing buffer 10x (100 millimolar (mM) Tris pH 8 ,
500 millimolar (mM) NaCl, 10 millimolar (mM) EDTA)
- 10 µL DNA oligos A and B (10 micromolar (µM) each)

4 Mix well and incubate for 00:05:00 at 90 °C

5m

5 Cool slowly to room temperature (aprox. 01:00:00) by removing the heat block from the apparatus

1h

6 Run agarose gel (1%) to check the annealing product

7 Digest pYCR with BsaI and inactivate the enzyme by PCR clean up

8 Ligate the annealing product with the precut pYCR:
Mix:

- 10 μ L of annealing product
- 60 ng of digested pYCR
- 1 μ L T4 ligase

- Transform the ligation mixture into E. coli MC1061 using the heat-shock method.
Grow the cells on LB agar plates + Erythromycin 0.1 mg/mL
- To select the transformants perform a colony PCR with Fw primer binding upstream of PvuII and Rv primer downstream SfiI cloning sites.

Expected result

Successful transformation will have a size of 427 pb

- Miniprep and sequence the constructs

Clone flanking regions into pYCR containing sgRNA

- Select the repair fragment of choice:
 - for knock-out: PCR amplify approx. 1 kbp upstream and downstream of the sgRNA targeted region
 - for knock-in: sequence of the desired insertion sequence flanked by SfiI restriction sites compatible with the backbone of pYCR
- Digest the pYCR(+sgRNA) and the repair fragments with SfiI
Inactivate SfiI by PCR clean up
- Ligate the components using T4 ligase
Heat inactivate the ligase (incubate 00:20:00 60 °C)

20m

- 15 Perform SmaI digestion to get rid of the original pYCR
- 16 Transform the ligation mixture into *E. coli* MC1061 using the heat-shock method.
Grow the cells on LB agar plates + erythromycin
- 17 To select the transformants perform a colony PCR with Fw primer binding upstream of PvuII and Rv primer downstream SfiI cloning sites.
- 18 Miniprep and sequence the constructs

Transform final construct in *B. mycoides* M2E_15

- 19 Transform using an electroporation protocol using 5 µL plasmid DNA

Protocol

NAME

Cloning of *Bacillus mycoides*

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PREVIEW

- 19.1 Pick 1 colony of *B. mycoides* M2E_15 and inoculate it in BHIS
- 19.2 When OD_{600nm} reached 0.85 add 2% glycine and 2% threonine in order to weaken the cell

wall.

- 19.3** Grow the cells overnight at 200 rpm, 30°C
- 19.4** Dilute the overnight culture 50 times in LBSP medium until the OD_{600nm} reaches 0.65
- 19.5** Collect the cells by 4000 x g, 4°C, 00:10:00 . Discard supernatant
- 19.6** Wash cell pellet with pre-chilled electroporation buffer (10% glycerol, 0.25 M sorbitol, 0.25M trehalose) x4
- 19.7** Suspended in 1 ml electroporation buffer (10% glycerol, 0.25 M sorbitol, 0.25M trehalose).
- 19.8** Snap-freeze the electrocompetent cells in liquid nitrogen and store at -80 °C
- 19.9** For the PCR mix pipette:
- 5 µL primers mixture Fw + Rv (5 uM) (see table "Primers")
 - 10 µL HF buffer
 - 1 µL dNTP (10mM)
 - DNA template (0.1 ng)
 - 0.25 µL phusion polymerase

- MiliQ to 50 µL

19.10 PCR reaction is performed with the following protocol:

Temperature (*C)	Time (mm:ss)
98	05:00
98	10:00
Variable	00:30
72	00:30
repeat above steps	30x
72	10:00
11	Infinite

19.11 Load 5 µL of sample plus 1 µL of staining solution 6x. Check the length of the band (bp).

Purify sample : PCR cleanup/ gel extraction according to manufacturer.

19.12 Digest the polymerized insert and the desired vector with 2U of appropriate restriction enzyme. See table "Primers" in order to choose the enzyme.

19.13 Digestion mixture (20 µL):

-Sfil 2 U

- Plasmid (50 ng)

-Insert (20 ng)

- 2 µL CutSmart 10x (or appropriate buffer)

-MiliQ to 20 μL

19.14 Incubate 01:00:00 at the temperature recommended by the manufacturer of the restriction enzyme

19.15 Inactivate the restriction enzyme by incubating 20 min 65 °C
**Sfil can't be heat inactivated so in order to inactivate it a PCR clean up is necessary

19.16 Ligate the digested insert and vector.
Ligation mixture :
-10 ul digestion product
-1 ul T4
-2 ul T4 ligase buffer
-7 ul MQ

19.17 Incubate 02:00:00 Room temperature

19.18 Thaw on ice 100 μL of electrocompetent cells

19.19 Add 2 μg of plasmid and the aliquot of electrocompetent cells to ice-cold electroporation cuvettes

19.20 Electroporate 25 uF, 10 kV/cm, 200 Ohms

19.21 Add 1 ml of BHIS and incubate 100 rpm, 30°C, 05:00:00 for recovery

19.22 Plate on LB + Agar (1.5%) + 100 µg/ml spectinomycin.
For crispr cloning, to activate cas9 expression, add 0.2% mannose.

19.23 Incubate at 30 °C Overnight

19.24 Randomly pick potential mutants and test by colony PCR (for primers use the ones that flank the region of insertion sites).

19.25 After selecting colonies that show the expected band size, purify the desired plasmid (miniprep) and transform into *B. mycoides*.

20 Plate on LB agar containing erythromycin
Grow at 28 °C overnight

21 Pick colonies and test them by colony PCR
If the expected band size is present, purify the plasmid and sequence

22 Grow the colonies with the expected sequence on liquid LB + erythromycin + manose
Incubate 200 rpm, 28°C, 12:00:00

23 Next day pick colonies in BHI medium (without antibiotic) and grow at 37 °C overnight

24 Dilute the liquid cultures and plate them on LN (no antibiotic)
grow overnight at 37 °C