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Fluorescent labeling *Bacillus mycoides*

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 The protocol is developed based on literature and has not been tested yet.

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ABSTRACT

GFP method is performed in bacteria to localize a desired peptide in the bacteria. In this experiment, we are focusing on the Nlp-14a. In the paper written by (Yi,2017) GFP was mutated on a *B. mycoides* E18 strain to study the plant-interaction studies. In their paper, they constructed a superfolder green fluorescent protein (sfGFP) and red fluorescent protein (mKate2) in the *B. mycoides* E18 strain. They tracked the GFP- tagged bacterial strain after inoculating in Chinese cabbage plants in a hydroponic system. They noticed that the bacterial strain rapidly attached to the plant during colonization and formed a matrix.

CITATION

Yi, Y., Frenzel, E., Spoelder, J., Elzenga, J. T. M., van Elsas, J. D., & Kuipers, O. P. (2017). Optimized fluorescent proteins for the rhizosphere-associated bacterium *Bacillus mycoides* with endophytic and biocontrol agent potential. *Environmental microbiology reports*, 10(1), 57-74.

LINK

[10.1111/1758-2229.12607](https://doi.org/10.1111/1758-2229.12607)

Strains and Growth conditions of bacteria

1 Routinely culture *Bacillus* strains in Luria-Bertani (LB) medium at 30 °C with aeration at 200 rpm.

2

Preparation of *B. mycoides* cells for GFP

3 Prepare the *B. mycoides* strain aliquotes for electroporation.

3.1 Cultivate the bacterial strain overnight in LB broth at 30 °C and at 180 rpm.

3.2 Transfer 1 mL of the overnight culture into 100 mL of LB medium (with 2% [wt/wt] glycine) and incubate it at 30 °C and 180rpm until optical density at 600 nm is 0.4 to 0.7.

3.3 Centrifuge the cells and wash the pellets with increasing concentrations of ice-cold glycerol (2.5%, 5%, and 10%). Resuspend this pellet in precooled electroporation buffer (10% glycerol) and shock freeze in liquid nitrogen.

4 Add the library vector DNA in an amount of 2 µg to the cells, and perform electroporation. The settings for electroporation are 2.0 kV, 25 IF and 200 X in a 2-mm cuvette using a Bio Rad Gen Pulser II electroporation system (Bio-Rad).

5 Add 1 mL of LB medium and grow the cells for 02:00:00 at 30 °C and 150 rpm for recovery and then plate on LB-Cm4 agar.

- 6 After 24:00:00 of growth at 30 °C , harvest the colonies from the plates and pool in LB medium
- 7 Store the libraries at -80 °C as 15% glycerol stocks.

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- 8 Inoculate the *B. mycoides* strain *mKate2mut* library in 50 mL of LB-Cm4 and grow at 7 or 6 to an OD_{600nm} of 0.3-0.6.
- 9 *B. mycoides* has been seen to show extensive cell-chaining and hence a mild sonication step of 4 rounds of 3 X 10 pulses of 1s with an amplitude of 30% can be applied to disassemble the aggregated cells.
- 10 Sort the cells on a flow cytometer at 20 psi using a 70 micromolar (μM) nozzle at a flow rate of 1.0 with the highest sort precision mode (0– 32-0 sort purity mask).
- 11 Using a sequential gating strategy with FCS height versus widths, followed by SCC height versus width, cellular debris, and chained cells can be excluded.
- 12 To separate the brightest variants choose a cutoff of 3% of the brightest event in the first round of cell sorting and 0.3% of the brightest events in the second round of sorting with the light scatter parameters.

Screening of FP variants and flow cytometry measurements

- 13 After FACS sorting, plate the final fluids containing bright cells on LB-Cm4 plates and grown them

overnight at 30 °C .

- 14** Observe the colonies using a fluorescence microscope. Keep the filter setting for GFP as excitation at 460/480nm and emission at 495/540 nm with a 485 nm dichromatic mirror; for RFP, the filter setting can be kept as excitation at 545/580 nm and emission at 610 nm with a 600 nm dichromatic mirror.
- 15** Capture the images on a camera and calculate the intensity of single-cell with Image J software.
- 16** Calculate the total cell fluorescence the formula is: Corrected total cell fluorescence (CTCF) = Integrated Density – (Area of selected cell x Mean fluorescence of background readings)