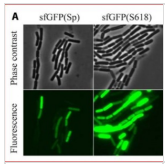

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Fluorescent labeling Bacillus mycoides

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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.

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. http://10.1111/1758-2229.12607

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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 lF 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.

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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 **pH7** or **pH6** to an OD_{600}nm 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 **[M]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)

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