**Created:** 9-25-22 by YZ

**Allelic Exchange in *Flavobacterium psychrophilum***

**Purpose:** This protocol describes the use of the PompA-*sacB* carrying suicide pYT313 (or pYT354) to generate an in-frame gene deletion (in gene “B”) in *Flavobacterium psychrophilum* wild-type strain (CSF259-93) by allelic exchange. This method can also be used to generate other gene replacements such as point mutations.

**Maps:**



**Map of genes ABC**. PCR products from P1 - P2 (upstream homology arm) and P3 - P4 (downstream homology arm) are cloned into the MCS of pYT313 to generate the deletion construct. The region to be deleted is between P2 and P3 in gene B. PCR screening to identify the deletion is performed with primers P5 and P6 or P7 and P8. P5 and P6 can be designed as primers used for complementation of gene B. P7 and P8 are located upstream and downstream of the homology arms on the chromosome. P7 and P8 are the most convincing primers to confirm the deletion but not suitable for PCR screening because of the long product.

P8

P7

A

B

C

P1

P2

P3

P4

sacB

pYT313

7610 bp

P6

P5

**Map of pYT313**. pYT313 is the *Bacteroidetes* suicide vector pLYL03 with PompA-sacBcloned into the EcoRI site (1).

**Procedure:**

1. **Primer Design:** For an in-frame gene deletion, design primers (P1, P2, P3 and P4) with the appropriate restriction sites (for cloning into pYT313) to amplify approximately 2 kb upstream and 2kb downstream of the region to be deleted. The sizes of homology arms can be longer or shorter than 2 kb, which depends on the species and genes to delete. Longer homology arms may allow DNA recombination to happen more easily.

**Note:** We normally don’t delete the intact gene B but leave the 5’-end (30-150 bp from the start codon, in frame) and 3’-end (30-150 bp from the stop codon, in frame) on the genome. This is to avoid affecting the transcription of gene A and gene C.

1. **Cloning:** Amplify the upstream and downstream homology arms and clone them into pYT313 step by step to generate the deletion construct. Typical restriction sites used are BamHI for P1, SalI for P2, SalI for P3 and SphI for P4. However, if these restriction sites are found within the sequence that is being amplified then other sites will need to be used. Cloning is done in the *E. coli* strain DH5MCR.

1. **Co-transformation**: The deletion construct and methylation helper plasmid (pSS05) (2) are transformed into the donor *E. coli* strain S17-1 lambda pir by heat shock.
2. **Conjugation:** Transfer the deletion construct from the donor *E. coli* strain S17-1 lambda pir to *F. psychrophilum* CSF259-93 by conjugation. Spot the conjugation mix on TYES agar and incubate at 18-20 °C for 2 d.
3. **First Recombination (plasmid integration):** Remove the conjugation spot from the agar with a sterile plastic spatula or wood stick and suspend in 1 ml of TYES broth. Prepare 600 µl of a 1:10 dilution of the cell suspension in TYES broth (10-1 dilution). Spread several TYES + erythromycin (20 µg/ml) plates with the undiluted conjugation mix (100 µl per plate) and several TYES + erythromycin plates with the 10-1 dilution of the conjugation mix (100 µl per plate). Incubate plates at 18°C for 7 days.
4. **STREAK COLONIES FROM FIRST RECOMBINATION FOR ISOLATION before proceeding to next step:** Pick one or more colonies from the 10-1 plates and streak them for isolation on TYES + erythromycin. If no colonies were obtained on the 10-1 plates then proceed with colonies from the undiluted plates. Incubate at 18°C to obtain isolated colonies. Cells should be erythromycin resistant and sucrose sensitive due to integration of the plasmid. Grow overnight in TYES containing erythromycin and freeze first recombination strain (**pure strain after isolation,** -80 C) in approx. 15% glycerol for storage.

**Note:** This step is extremely important. Colonies shown on erythromycin plates after step 5 may also contain live wild type *F. psychrophilum* and *E. coli* cells in addition to the real conjugant cells (with plasmid inserted to the chromosome). Growing this mixture without antibiotics will cause unpredictable results and failure in the next steps.

1. **Second Recombination (loss of plasmid and region of interest):** Inoculate 3 ml of TYES with an isolated colony from step #6 in the ABSENCE of erythromycin to allow for the second recombination and loss of the plasmid. Incubate overnight at 18°C with shaking. Prepare 10-fold dilutions of the overnight culture to 10-3 in TYES broth and plate 100 µl of each dilution on TYES agar containing 2.5% (w/v) sucrose (2). Incubate plates at 18°C for 7 days. Isolated colonies are normally obtained on 10-1 to 10-2 diluted plates.
2. **Selection of recombinants from step 7.** Patch the same single colonies (at least 20) obtained from step 7 on both sucrose and erythromycin plates. Incubate at 18°C. The recombinants that have lost the plasmidshould not grow on erythromycin but grow on sucrose plates.
3. **PCR Screening:** Theoretically, loss of the plasmid in the second recombination event should result in 50% of cells carrying the wild-type allele and 50% of cells carrying the mutant allele. To identify cells carrying the mutation use primers flanking the deleted region (see map: P5 and P6) and perform colony PCR using colonies that failed to grow on erythromycin plates from step #8. **PCR Controls:** For a wild-type allele control use wild-type chromosomal DNA or a wild-type colony lysate as the template and for a mutant allele control use the deletion plasmid construct generated in step #2 as the template.

**Note:** Primers P1 and P4 used to clone the upstream and downstream homology arms can also be used for PCR screening and amplifying across the deleted region. However, this results in a much larger PCR product. If additional verification is desired, a PCR screen using a primer internal to the deletion can be used. This will result in no amplification from cells carrying the deletion, while a positive control with wild-type cells will result in amplification.

1. **Confirm deletion:** Purify the sucrose resistant mutants (erythromycin sensitive) by streaking and confirming the deletions again using PCR. For absolute confirmation could perform Southern blot, or perform PCR confirmation with primers outside of P1 and P4 (P7 and P8) and sequence the product. This avoids false positives from amplification off of plasmid for example, and requires that the plasmid integrated and then was lost.

**References**

1. Y. Zhu *et al.*, Genetic analyses unravel the crucial role of a horizontally acquired alginate lyase for brown algal biomass degradation by *Zobellia galactanivorans*. *Environmental microbiology* **19**, 2164-2181 (2017).

2. S. Sloboda *et al.*, Methylation of foreign DNA overcomes the restriction barrier of *Flavobacterium psychrophilum* and allows efficient genetic manipulation. *Applied and environmental microbiology* **Under Revision** (2024).