Transfer of exogenous DNA (plasmid) to *Vibrio* spp. by conjugation

Introduction

Many environmental strains of *Vibrio* are not transformable by classical techniques (chemicompetence, electroporation, chitin), and are therefore genetically manipulated by conjugation. This involves transferring a plasmid from an *E. coli* donor strain to a recipient *Vibrio* strain.

The plasmid contains:

- **A replication origin, oriV**, constitutive or conditional depending on the objectives.
  - We use two types of replicative plasmids: oriV-p15A has a broad bacterial spectrum; oriV-pMRB is specific to and stable in *Vibrios*. These plasmids are used to express a fluorochrome (e.g., GFP) in *Vibrio* or for complementation experiments. When constitutively expressing a gene, it is cloned under the control of a P_{Lac} promoter; when controlled expression is desired, the P_{BAD} promoter is used.
  - We use a conditional (or suicide) vector whose replication is mediated by a protein (Pir). The gene encoding this protein is present in the conjugation donor but absent in *Vibrios*. This suicide vector is used for recombination-mediated integrations into the genome to inactivate or delete a genetic element. Mariner-type transposons can also be cloned into it to generate random insertion libraries.

- **A transfer origin oriT** compatible with the conjugation system expressed by the donor, we use the oriT RP4 system.
- **A selection marker,** a gene conferring resistance to an antibiotic.

The *E. coli* conjugation donor strain (named beta XXX) contains:

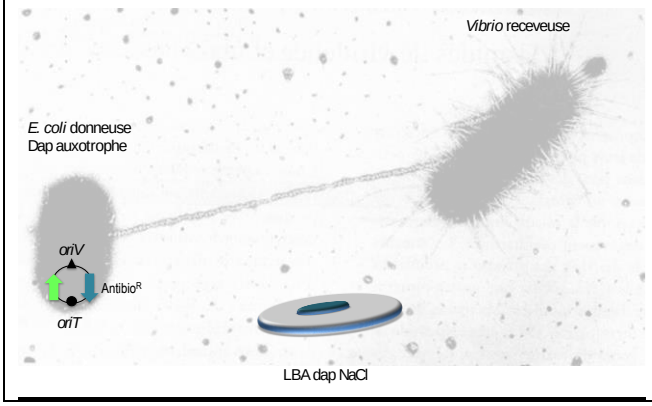
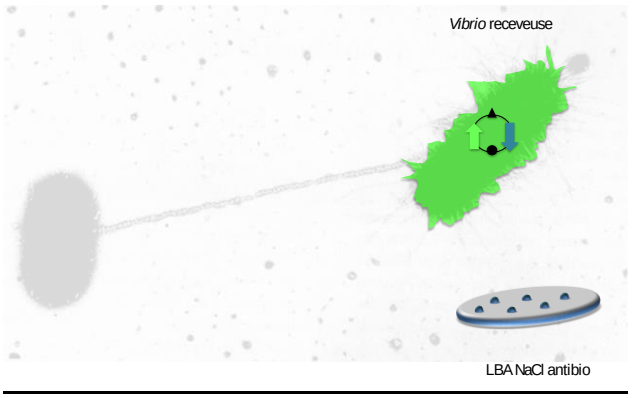
- The conjugation genes of the RP4 system coupled with the kanamycin resistance gene.
- The pir gene to allow replication of the suicide vector.
- It is auxotrophic for DAP (diaminopimelate) for its elimination (counterselection) after the conjugation and plasmid transfer to the *Vibrio* strain.

Note that competence in this beta strain is not optimal. Therefore, our cloning is carried out in another strain (named Pi XXX), where constructions are verified, and plasmids are transferred to the donor. The Pi strains contain:

- The pir gene to allow replication of the suicide vector.
- It is auxotrophic for dT (Thymidine).

The recipient *Vibrio* strain may be more or less resistant to antibiotics (in our hands, usually only chloramphenicol and spectinomycin are usable). They may be more or less permissive to the transfer of exogenous DNA (due to the numerous defense systems in *Vibrios*). Therefore, it is necessary to perform an antibiogram in advance and test several strains of interest to select the best recipient (if possible). Tips at the end of this section help improve the conjugation frequency, which can vary from 10^{-1} to $<10^{-7}$ depending on the strains.

Schematic summary :

Conjugation: Compromise medium and temperature between *Vibrio* and *E. coli*.	Selection of *Vibrio* cells that have acquired the plasmid, counter-selection of *E. coli*.
	

Materials (example for V. crassostreae)

- Marine broth (MB)
- Marine agar (MA)
- Lysogeny broth (LB) Miller
- LB + agar (LBA)
- NaCl 5M
- DAP 50mM (SIGMA 33240-5G)
- Chloramphenicol (Cm) 25 mg/mL
- LB NaCl 0.5M
- Kanamycin (Kan) 100 mg/mL
- TSA-2 (BBL Trypticase Soy Broth (TSB), BD, ref. 211768). Pour 1L de TSA-2: TSB 30 g; NaCl 15 g; Agar 15 g; H_20 necessary quantity for 1L
- Conjugation TSA-2 + DAP 0.3 mM **ou** LBA NaCl 0.5M + DAP 0.3 mM
- Selection : TSA-2 + Cm 5ug/mL **ou** LBA NaCl 0.5M + agar+ Cm 5ug/mL

Bacterial culture

E. coli beta: LB DAP 0.3mM, 37°C, 250 rpm

Vibrio: MB, 20°C, ou LB NaCl 0.5N, gentle agitation (100 rpm)

37°C incubator for E. coli

30°C incubator for conjugation

20°C incubator for *Vibrio* and selection

Protocol:

Day -2

Thaw the cells on agar:

- *Vibrio* on MA.
- Conjugation donor on LBA+ DAP 0.3mM + Kan100 ug/mL + antibiotic encoded by the plasmid, here Cm 25 ug/mL.

Day -1

Start 5mL of overnight culture:

- *Vibrio* in MB.
- *E. coli* donor in LB DAP 0.3 mM+ Cm25 ug/mL (Kan is not added in liquid phase).

Day 0

Dilute the cultures 1/100 in 5mL

Vibrio: LB NaCl 0.5M, 20°C, gentle agitation.

E. coli: LB DAP 0.3 mM (no antibiotic), 37°C, 250 rpm.

Monitor growth (OD) regularly. At OD 0.3, mix 1mL of *Vibrio* with 5mL of *E.coli* donor, centrifuge at 6000 rpm for 10 minutes, remove most of the supernatant (quickly invert the tube onto absorbent paper) and resuspend the pellet in minimal volume (25ul) of LB NaCl 0.5M.

Place the mixture on a well-drained TSA-2 DAP 0.3 mM agar plate (the deposit should be very thick and small) and incubate overnight at 30°C.

Day 1

Scrape the biofilm/spot thoroughly with a pipette tip (P1000) to collect everything.

Place the pipette tip with the bacterial pellet into a 15mL tube containing 2mL of LB NaCl 0.5M, and vortex vigorously for 5 seconds to fully resuspend the cells.

Plate pure and/or diluted TSA-2 Cm5 ug/mL selection agar; alternatively (when the conjugation frequency is good, i.e. 10^{-3} minimum) deposit 10 ul of suspension and streak for isolation on plates.

Incubate for 24 to 28h at 20°C depending on *V.crassostreae* strains.

The ratio between the number of conjugants (selected on antibiotic) and the total number of colonies of *Vibrio*s obtained (agar without antibiotic) gives you the conjugation frequency.

Subculture a few clones onto a selective LBA medium containing 0.5 M NaCl and 5 µg/mL chloramphenicol. Incubate at 20°C overnight. Confirm the presence of the plasmid by performing a miniprep or PCR.

Important tips!

It's essential to understand that each *Vibrio* strain has its own characteristics: possible antibiotic types for selection, permissiveness to exogenous DNA, etc. Therefore, we provide some advice here to start a genetics project with your preferred strain.

- Aim to have a **collection of strains** rather than a single strain to manipulate. Determining if your strain is genetically modifiable (the most genetically modifiable) may be something to ascertain before sequencing it.

- Test the **resistance of your strains** to commonly used laboratory antibiotics (and resistances carried by your plasmids), generally chloramphenicol, spectinomycin, ampicillin, and kanamycin.

- *Vibrio*s spp. inhabit various ecological niches. Breton *Vibrio*s are cultured at 20°C, Norwegian *Vibrio*s at 16°C, and New Caledonian *Vibrio*s at 28°C. These **parameters should be considered for liquid culture and conjugation**. For example, cold *Vibrio*s will die at 30°C, so plan for conjugation at a lower but longer temperature.

- Conjugation and selection can be carried out on LBA NaCl 0.5N instead of TSA-2, depending on the strains. The initial reference cited exclusively uses LB and derivatives.

- Conjugation generally occurs with *Vibrio*s in exponential growth phase, but some prefer higher OD (0.9).

- **Antibiotic selection is also variable**; for example, *V. crassostreae* is selected on Cm 5 µg/mL, while *V. nigripulchritudo* and *V. aestuarinus* are selectable on Cm 1 µg/mL.

- When conjugation frequency is high, a "rapid" alternative is to directly pick the bacteria who are donors and recipients from the agar, mix them with a pipette tip on the conjugation plate TSA-2 DAP 0.3mM, and incubate (i.e., without going through a liquid culture step); then after an overnight incubation at 30°C, pick the biofilm/spot and resuspend it in LB NaCl 0.5M.

- Conversely, when the conjugation frequency is low, one may need to perform 10X a mixture of 10 mL donors and 1 mL recipient, make 10 drops of biofilm/spot, and select on a large number of plates.

- When resuspending the biofilm/spot in 2 mL of LB NaCl 0.5M and spreading the pure mixture, a significant background noise can occur, so it's necessary to dilute it for the selection step.

- Some strains are poorly permissive to replicative plasmids transfer (low to no frequency) but accept suicide plasmids, thus remaining genetically modifiable (see protocols for mutation by integration and mutation by allelic exchange).

Reference

Le Roux F, Binesse J, Saulnier D, Mazel D. Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. **Appl Environ Microbiol.** 2007 73(3), 777-84.