Inactivation or deletion of genetic elements (including plasmid curing)

Introduction

Formal demonstration of the function of a gene or genetic element (genomic island, plasmid etc.) requires its inactivation or deletion to show that it is essential to the phenotype. We use a suicide plasmid, which can replicate in the donor E. coli strain but not in Vibrio. This plasmid is transferred to Vibrio by conjugation.

The plasmid contains:

- a **conditional origin of replication, oriV**, whose replication is mediated by a protein (Pir). The gene encoding this protein is present in the conjugation donor but absent in the vibrio. This suicide vector is used for recombinant integration into the genome to inactivate or deletion of a genetic element. It can also be used to clone mariner transposons to generate allelic insertion libraries.
- We use the oriT RP4 system, an **oriT transfer origin** compatible with the conjugation system expressed by the donor.
- A **selection marker**, an antibiotic resistance gene.

In the case of integration by simple recombination, this plasmid is cloned:

- **A 500 bp insert located:**
  - in the middle of a gene if you want to inactivate it
  - at the end of a gene if you want to introduce a marker (e.g. antibiotic resistance) into the genetic element without inactivating the gene.

In the case of allelic exchange (or POP IN-POP OUT), the suicide vector which also contains:

- **araC_pBAD_ccdB**: a bacterial toxin dependent on a conditional promoter, repressed by 1% glucose; activated by 0.2% arabinose.
- **A fusion insert 500 bp** upstream and 500 bp downstream of the gene to be deleted or exchanged.

The E. coli cloning strain (named Pi 3814) contains:

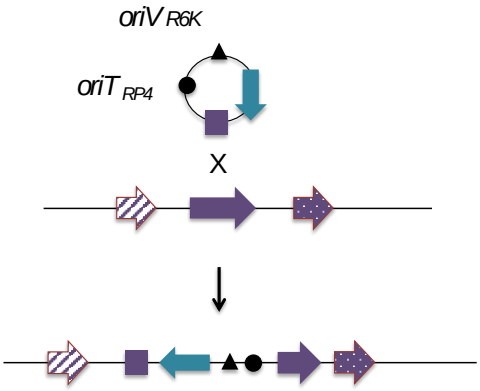
- pir gene for suicide vector replication
- It is auxotrophic to dT (Thymidine)
- It has a gyrA mutation that makes it resistant to ccdB.

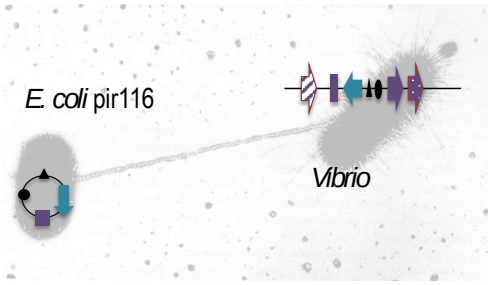
The E. coli conjugation strain (named Beta 3914) contains:

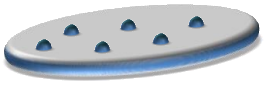
- RP4 conjugation genes coupled to the kanamycin resistance gene
- pir gene for suicide vector replication
- It is auxotrophic to DAP (diaminopimelate) to eliminate it (counter selection) after the conjugation step and transfer of the plasmid to the vibrio.
- It is also resistant to ccdB (gyrA mutation).

Graphical summary:

1- Integration of the suicide vector by one recombination event





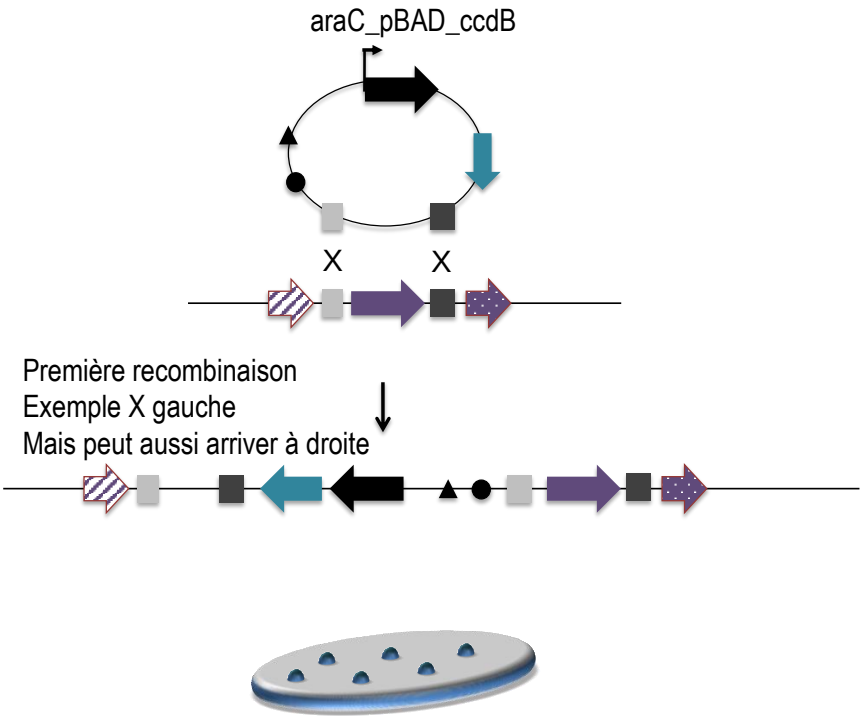


LBA NaCl antibio

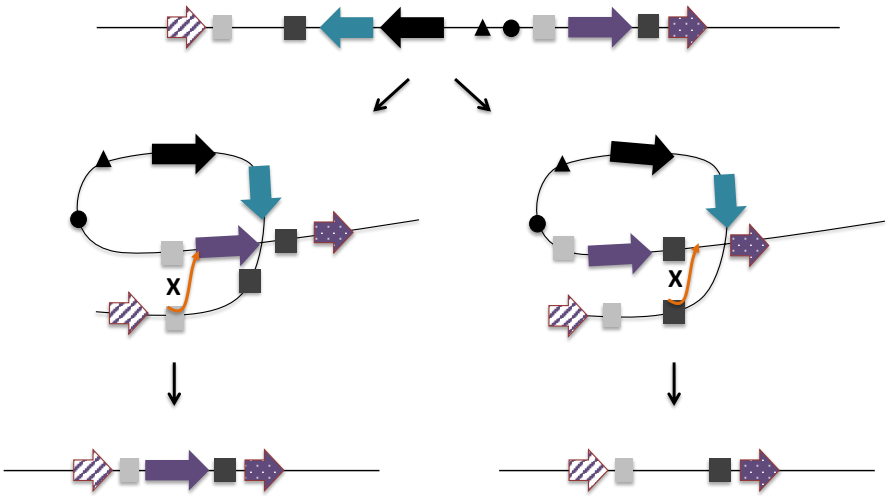
Advantages: rapid technique (2 days)
Disadvantage: the vibrio is then resistant to the antibiotic, and multiple inactivations cannot be performed. In addition, integration of the suicide vector can alter the expression of other genes in this region (polar effect). Generally speaking, when we observe the loss of a phenotype in a mutant, we must always demonstrate that complementation of this mutation restores the phenotype. This can be achieved by expressing the gene in a plasmid or by integrating it into the genome.

2- POP In POP OUT

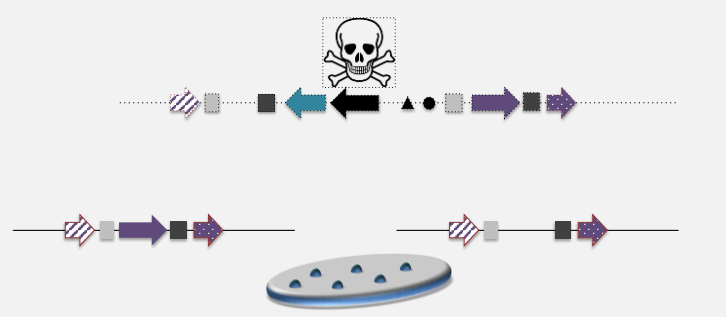
Step 1



Step 2: integration of the suicide vector is selected by the antibiotic. Glucose prevents ccdB expression. This integration generates a duplication of the flanking regions (light/dark gray X) which can then give rise to a second recombination.



Cells that have not undergone this second recombination event are counter-selected by activating ccdB (arabinose) expression; cells still carrying the suicide plasmid die. Mutates are selected by a deletion screen using PCR primers external to the deletion.



Advantages: you can accumulate as many deletions as you like, since counter-selection leads to the loss of the antibiotic resistance gene. In theory, deletion or allelic exchange does not modify adjacent genes.
Disadvantage: more time-consuming technique (5 days).

Material (example for V. crassostreae)

- Marine broth (MB)
- Lysogeny broth (LB)
- LB + agar (LBA)
- NaCl 5N
- Dap 50mM (SIGMA 33240-5G)
- Chloramphenicol (Cm) 25 mg/mL
- LB NaCl 0.5N
- Kan100 mg/mL
- TSA-2 (BBL Trypticase Soy Broth, BD, ref. 211768). For 1L de TSA-2: TSB 30 g; NaCl 15 g; Agar 15 g; H_20 quantity necessary 1L
- Conjugation TSA-2 + dap 0.3 mM **or** LB NaCl 0.5N + agar + dap 0.3 mM
- Selection : TSA-2 + Cm 5ug/mL **or** LB NaCl 0.5N + agar+ Cm 5ug/mL
- In the case of allelic exchange : recombination first selection TSA-2 + Cm 5ug/mL + Glucose 1% **or** LB NaCl 0.5N + agar+ Cm 5ug/mL+ Glucose 1% ; counter selection second recombination: LB NaCl 0.5N + agar+arabinose 0.2%.

Bacterial culture

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

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

37°C incubator for E. coli

30°C incubator for the conjugation

20°C incubator for vibrio and selection

Protocol:

Day-2

Thaw cells on agar:

- Vibrio on MA

- Conjugation donor on LBA+ dap+ Kan100+ plasmid-encoded antibiotic, here Cm 25 ug/mL

Day-1

Launch 5mL of culture overnight

- Vibrio in MB

- E. coli donor LBA dap0.3 mM+ Cm25 ug/mL (no Kana added in liquid phase)

Day 0

Dilute cultures 1/100 in 20mL

Vibrio: LB NaCl 0.5N, 20°C, gentle agitation

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

Monitor growth (OD) regularly. At OD 0.3, mix 2mL of vibrio with 10 mL of donor E. coli, centrifuge at 6000 rpm for 10 minutes, remove as much supernatant as possible (quickly invert the tube onto adsorbent paper) and recover the pellet with a minimum volume (25ul) of LB NaCl 0.5N.

Deposit the mixture onto well-dried 0.3 mM TSA-2 Dap agar (the deposit should be half a millimeter in size), and incubate overnight at 30°C.

Day 1

Scrape off the oily biofilm/spot with a tip (P1000) to recover everything

Place the tip with the bacterial pellet in a 15mL tube containing 2 mL LB NaCl 0.5N, and vortex vigorously for 5 seconds to resuspend the cells.

Spread 500 ul onto a large selection agar plate; depending on the strain and gene targeted, one or more plates are required.

- For single recombination integration, select on TSA-2 Cm5

- For allelic exchange, select TSA-2 Cm5 Glucose 1%.

Incubate for 24 to 48 hours at 20°C, depending on the V. crassostreae strain.

In the case of single-recombinant integration, confirm integration at the correct site by PCR using a primer in the plasmid and a primer in the bacterial genome.

In the case of an allelic exchange, continue with the second recombination for this :

Day 2

Select 4 clones on TSA-2 Cm5 Glucose 1% and culture them in LB NaCl 0.5N Cm5 for 6-8 h.

Spread 10 (isolation) and 100 ul (rake) on LB NaCl 0.2% arabinose.

Incubate for 24 hours at 20°C.

Screen mutants by PCR using external primers

Important tips!

It's important to understand that each vibrio strain has its own particularities: type of antibiotic available for selection, permissiveness to exogenous DNA, etc. In addition to the advice given in the conjugation protocol, here are a few tips for integration:

- Cumulative conjugation/integration frequencies vary greatly according to strain and target. For V. crassostreae, we generally isolate several meriploides on one dish (500 ul selection of conjugation juice); for V. aestuarianus, we need 10 to 20 large dishes (5 conjugations, taken up by 2 mL = 10 mL , 500 uL spread over 20 dishes.).
- The frequency of integration of the suicide plasmid depends on the size of the region used for recombination, generally 500 bp, but can be reduced to 150 bp if necessary.
- Some strains are non-permissive to replicative plasmids but permissive to the integration of a suicide vector, so don't despair!
- Some people tend to add glucose to the conjugation agar, but don't! Glucose has a negative effect on E. coli, which no longer conjugates. Glucose is only used for the selection of conjugating vibrios.
- For genomic islets (in particular those involved in antiphage defense), it may be necessary to perform subdeletions in order to deletion the complete islet; as with plasmids, deletion of the toxin of an addiction system can also be used to perform total deletion.
- The same tools are used for curing plasmids, to:
    - Clone the origin of replication of the vibrio plasmid into the suicide vector (without ccdB), transfer it into the vibrio strain and select on antibiotics. The endogenous plasmid and the transferred plasmid enter into competition (incompatibility linked to the same origin of replication) and strains that have lost the endogenous plasmid are selected (Le Roux NAR 2011).
    - Clone 500 bp of the endogenous plasmid into suicide plasmid encoding ccdB, select for integration by recombination on antibiotic/glucose, then culture in arabinose to counter-select plasmid loss (Bruto ISME J 2017).

References

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.

Le Roux F, Davis BM, Waldor MK. Conserved small RNAs govern replication and incompatibility of a diverse new plasmid family from marine bacteria. **Nucleic Acids Res**. 2011 39(3), 1004-13.

Bruto M, James A, Petton B, Labreuche Y, Chenivesse S, Alunno-Bruscia M, Polz MF, Le Roux F. *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. **ISME J.** 2017 11(4), 1043-1052.