Conjugation of Thalassiosira pseudonana

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Abstract

This protocol was used to express a gene encoding a silaffin precursor TpSil3p-APEX2 fusion protein in *T. pseudonana* strain CCMP1335 via conjugation largely following Karas et al. (2015). In addition to the protocol I am listing some of my observations working with this diatom species. Please check the 'Before start' and 'Guidelines' sections.

1. B. J. Karas *et al.*, Designer diatom episomes delivered by bacterial conjugation. *Nat. Commun.* **6**, 6925 (2015).

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Guidelines

[1] 'Crashing phenomenon' in *T. pseudonana* cultures

In my experience *T. pseudonana* CCMP1335 has to be subcultured frequently - every ~2 weeks - to avoid 'crashing'. I usually dilute my cultures 1:10 in fresh L1 medium. By 'crashing' I mean sudden take over of what I believe is a naturally associated bacterium in the culture. This usually happens in the stationary phase over the course of 1 or 2 days during which brown diatom culture becomes milky due to bacteria. It could be a standard contamination issue, but I've pretty consistently observed this phenomenon even after ordering completely fresh stocks from the NCMA. I analyzed the bacterium via 16S sequencing and some simple growth experiments and confirmed it was a *Pseudomonas* species. You can read more about how I've done this here.

Interestingly, this *T. pseudonana-Pseudomonas* interplay was already observed in the late 1970's.

[2] Episomes for *T. pseudonana* conjugation available upon request

All episomes used in my conjugation experiments including their maps are available upon request. Send an email to turnsek@fas.harvard.edu, turnsek@ucsd.edu or jturnsek@jcvi.org and we'll go from

there/I can provide more information. Here is a list of available episomes:

pTpPuc3 - empty nourseothricin resistance cassette-containing episome for *T. pseudonana* conjugation

eGFP - 2 different enhanced GFP constructs

DreidelTeal - blue chromogenic protein

TpSil3p-eGFP - frustule-associated silaffin precursor protein TpSil3p tagged with eGFP

TpSil3p-APEX2-FLAG - frustule-associated silaffin precursor protein TpSil3p tagged with APEX2 and FLAG

TpSil3p-APEX2-6xHis - frustule-associated silaffin precursor protein TpSil3p tagged with APEX2 and 6xHis

NOTE:

All the genes above were cloned into pTpPuc3 backbone via Gibson Assembly starting 36 nucleotides downstream of the HIS3 gene. They are flanked by a constitutive fcp promoter and an associated terminator. Here are their sequences in FASTA format:

>Tp_fcp_promoter

GCGCTTTTTCCGAGAACTCCCCATAAGTCAACGGCTCCAATCAAGAATGTATCCGACAACGGCGAGCATAGCAA
CACGTCCGTCTTTGGAGTAGAATCATCATGTTGTGGATGAATACACAGA

TGAATGACATTAAAAGCATGAACATGTTAGAGAGTAGGAGGTAGAGATTGATATGGTAGCATTGCGATGTTTGT
TTTTGGTCAGCATATGATGAGTGGATACCAATATGATGAAAGTTGAATCT

CGCGTTTGAGCTCAGCGGTACGTTATTGATCGAAAGTAGCCTGATCAAAATCCTTGGAGAGTACAAGAGGATCA
AAGAATCCAGTGGGGGCGATAACTCCAAGCTCGTTCTCAAAGAGGCAA

TGGAGGTAGAAACTCATCCCAGTTGAGAAGAAGTGAAGGCAGTGGCGGTGGCGAAAGCAGAGGCAACGAGGA
CAGACTTCCTGTGGGTTGATGCAACGAATATTTCCAGAAGGAGAAG

TTTAGAGAGTTGAACCGCTACCTACAATGACAAAGTATCGTATCGATTTTGATGTTGGTTGGTTATGAATTCAAA
CTGTAAGTTGGATTGTGAGAAGATCAGAAGTTGAACGAACACATCTTTC

CGATCATTCACCTCCACACTGCAACAACACGGTACTTCTTCCGCGGCAGGTCTCTGTCGCCATTCTCTTGTCCTG
TTGTTGGCTGTGAGACGAGGAAAGCAACGACAAGTTTCACAAAAGGG

AGTTCCTTTAACGAGATATGTTTTTTATAAAGAGTCCCAATAGAAAGACAAATTGATTCCTCCGTGCAAACGCGC
AAATAAACACCACGTCCATTATATCCATATCTTTCAGAGTATCCAACAAGT

GTTGAAGGACAGGTAGTTGAAGTAACGTATCTTCCCCCTCGACTGGATCCATCAACAAGGCGAACAAATCCATT
CAACCTCTCATAAATTATCTGATTTACCAAACC

>Tp_fcp_terminator

ATACTGGATTGGTGAATCAATGAGCCGTAGCACAATGGTTACATTCGGCTAGCTAAGATCCAATGGCAAGGACC
AAGTGCTGGAACTTGTTTTGCTTTAGCAGATCTTAGCGTGAGAGGTATT

TGTCCTCTGTCAGGAGTAGATAGTAGATGTTCTTTTTAAACTAAAATGCTAACTGTTCCGAATTCCTCATCGCAGC
TAATCCGTACATCAAAAGACAAAATGCTAGGTATGTGTACTACATCTCC

TGTTGCTAGATAAGACATATGATAGGAAACACACCATCAATAGTCATTGTAGCTTTACTTATACTACGCATTTGCA
CTTTCCCCTGAGTGGCAGAGGCGCATTGAGAAAATCGATCTCAACATAG

TTTATGTAGCATCCCCTAGATCCATTACTTTAAGTCTCCTTCGTCTTTGGTGTAGGCATGTTGGACACAACGAGG
TAAAACACAACACAAACAATGTGTCCAGCAAAGTAGTAGCTGCTCCAGT

TCT

Before start

Preparation of donor bacteria

1. Any *E. coli* strain will probably work just fine, but I stuck with the one from the original publication: EPI300 (Epicentre, catalog # EC300110).

2. Electroporate pTA-Mob into EP300 cells and select on LB agar plates w/ 10 or 20 µg/mL gentamycin.

3. Make pTA-Mob-containing strain chemically competent.

4. Tranform your engineered pTpPuc3 cargo plasmid into pTA-Mob-containing cells.

5. Select double transformants on LB agar plates with 10 or 20 µg/mL gentamycin and 50 µg/mL kanamycin.

6. Store plates at 4 ^{0}C.

Preparation of L1 medium and L1 1% agar plates

I was using L1 Medium Kit and Gulf of Maine Seawater, both from NCMA, to make my liquid medium and plates.

L1 medium

I would usually make batches of 1L by combining seawater, N, P and Si sources from the kit followed by autoclaving, cooling down to ~50 ^{0}C and adding vitamins and antibiotics. The resulting medium had pH ~8.5. Before starting a diatom culture I would adjust the pH with HCl to ~8.0.

NOTE:

The resulting medium contain some precipitates. You can use a combination of microwaving and filter sterilization instead which should prevent precipitation.

1/2xL1 1% agar plates w/ 5% LB

I would always prepare 0.5L and make ~12 plates 40 mL each. For 0.5L combine and mix seawater, N, P and Si sources from the kit, add agar and 5% LB. Autoclave, cool down to ~50 ^{0}C and add vitamins.

NOTES:

- 5% LB is **v/v not w/v**. I had success preparing plates w/v, but **v/v is what you should use**.

- 1/2xL1: cut all N, P, Si and vitamin volumes from the kit in half.

- I had success with Bacto Agar and Sigma's high purity agar.

1/2xL1 1% agar plates w/ 100 µg/mL nourseothricin

Same as above except the LB part; add nourseothricin when cooled down to ~50 ^{0}C.

NOTE:

I observed a lot of background - false positive - colonies on plates with only 50 µg/mL nourseothricin so I switched to 100 µg/mL. You can read more about nourseothricin on the following website which I found very useful.

Protocol

Step 1.

Growth and preparation of *E. coli* donor

1. Pick colonies from your Gent+Kan plates & inoculate 10 mL LB medium. Grow overnight.

NOTE:

Start growing colonies early in the afternoon the day before conjugation to get enough biomass. 16-20 hrs should suffice. Expect OD_{600} 2 next morning.

2. Measure OD_{600} and start a 150 mL LB subculture (starting OD_{600} either 0.05 or 0.1).

3. Grow at 37 C until OD_{600} reaches 0.3-0.4.

4. Centrifuge at 4000 rpm | 10 ^{0}C | 10 min.

5. Decant supernatant and resuspend in 800 µL SOC.

Step 2.

Growth and preparation of *T. pseudonana* cells

1. *T. pseudonana* was cultured in L1 medium with moderate shaking (130 rpm), constant light and 18 ^{0}C. L1 medium described in 'Before start' section.

NOTES:

- I've never tested if cell density before spinning cells down matters with respect to final conjugation outcome same as the Alverson Lab did for *P. tricornutum*. They observe 8 x 10^6 cells/mL to be a sweet spot. In my hands spinning cells down at 4-8 x 10^6 cells/mL seemed to work fine. According to my information harvesting *T. pseudonana* at 0.8 x 10^5 cell/mL works best for biolistic experiments.

- I counted cells with BioRad's TC20 automatic cell counter.

2. Spin down 2 x 10^8 cells at 4000 rpm | 10 ^{0}C | 10 min.

3. Decant supernatant and resuspend pellet in 1 mL L1 medium.

Step 3.

Conjugation

1. Mix 200 µL *T. pseudonana* cells and 200 µL *E. coli* cells in a 1.5 mL tube.

2. Pipette up and down a few times.

3. Plate on 1/2xL1 1% agar plates w/ 5% LB.

NOTE:

Make sure the plates are dry. Leave them open at 37 ^{0}C for an hour or so before plating diatom-bacteria co-cultures.

4. Incubate in dark at 30 ^{0}C 90 min.

5. Move plates to standard diatom growth conditions - 18 ^{0}C and constant light - for 4 hrs.

6. Add 1 mL L1 medim and scrape with a cell scraper or L spreader.

Step 4.

Selection

1. Expect to recover 500 µL co-culture suspension after scraping.

2. Plate 200 µL of the resulting suspension on pre-dried 1/2xL1 1% agar plates w/ 100 µg/mL nourseothricin.

3. Leave at 18 ^{0}C and constant light until colonies appear - 2 weeks.

Step 5.

Screening

Here are a few ways to confirm the presence and expression of your heterologous gene in resulting colonies:

1. Growth under selection pressure.

Make sure colonies are able to grow in liquid L1 medium with 100 µg/mL nourseothricin (Nou100). Pick colonies with a small tip or better a toothpick and inoculate 0.5 mL L1 medium. Once you observe growth subculture in larger volume.

2. PCR

- Use 1 µL of diatom culture as a template to amplify your expression cassette.

- Confirm the absence of donor DNA by amplifying *E. coli*-specific genes.

- Confirm the absence of live donor cells by plating some diatom culture on LB plates w/o antibiotics.

NOTE:

Any remaining donors cells and donor DNA are gone after a few liquid subcultures.

3. RT-PCR

- Purify total RNA from Nou100 diatom culture, convert it to cDNA and use it to run a PCR with heterologous gene-specific primers.

- Example of a result with controls can be found here.

4. Episome recovery

- Perform a diatom miniprep as described by Karas et al.

- Transform *E. coli* with diatom-derived DNA.

- Select on LB agar plates with 50 µg/mL kanamycin.

- Miniprep, digest and analyze on a gel.

5. Western blot

6. Protein-specific assays

- enzymatic assay

- microscopy

- ...

I've had success with 1, 2 and 3, tried 4, 5 and 6 without success.