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Abstract

Here we describe our efforts to uncover the life cycles of the microalgal model species *Phaeodactylum tricornutum, Emiliania huxleyi,* and *Ostreococcus tauri* at SZN, MBA and UPMC.
For *P. tricornutum,* we unsuccessfully attempted to isolate new strains from the field. In addition, we have incubated the mixed cultures of several strains under conditions believed to favour sex. These efforts remained without issue.
In *E. huxleyi* we attempted to induce sexual reproduction by inducing cross-fertilization of haploid gametes of *E. huxleyi*, expecting to find diploid cells. The unexpected complexity of the *E. huxleyi* life cycle and potential for decoupling ploidy level from morphology has revealed a more complex scenario than expected.
In *O. tauri* we attempted to induce sex between modified haploid strains with different antibiotic resistance and to screen the offspring for resistance to both antibiotics. Only false positives were obtained in the experiments performed.
Results obtained so far enrich our knowledge of the process under investigation and represent a solid ground for future attempts.

Table of Contents

1 Introduction

Phaeodactylum tricornutum (Pt)

Pt is a pennate diatom that occurs in three distinct shapes: triradiate, minute-oval and elongate-fusiform (Barker 1935). Its single chloroplast per cell renders it recognizable from the multitude of other pennate diatoms. Its defining feature constitutes its cell wall elements: those of most other diatoms consist of silica reinforced with an organic matrix, whereas those of Pt consists solely of organic material; only the fusiform shaped cells possess a single siliceous element covering only part of the cell. For this reason, Pt has been chosen as a model species for a range of research projects. The content of the living, including its genome, can be easily manipulated without having to penetrate the silica armour. Moreover, cultures can be kept indefinitely, which is important for the preservation of strains with desired characteristics, which in its turn is a prerequisite for sustained industrial applications.

One of the prerequisites for the use in research and industry is the control of a strain sexual cycle. To maintain strain integrity one needs to avoid sexual reproduction as it can lead to loss of desired combinations of traits. Yet, controlled selective breeding is the most straightforward way to obtain those desired combinations of traits. So, we need to know the phenology of the sexual cycle, and how it can be stimulated or blocked. Unfortunately, none of this is known.

Other pennate diatoms usually are dioecious, which is beneficial for abovementioned applications because it allows a controlled breeding programme and prohibits sexual reproduction in single strains, i.e., ensures strain integrity. In most pennate diatoms, sex requires two strains of the opposite mating type. Another characteristic of most pennates is that following sexual reproduction the offspring needs to "mature" for a given time or number of mitotic divisions before they become sexually mature. This constraint is correlated to cell size, but since cells of Pt are not encased in a rigid silica cell wall, this constraint may not count for Pt. Sex is usually taking place during optimal conditions as it requires considerable resources. Sexual reproduction has been described in several pennate species (e.g. D'Alelio et al. 2010). It involves pairing of cells of opposite mating type, meiosis and formation of usually two isogametes per cell, one pair moving over to the pair in the other cell, fusing with their counterparts, and the resulting zygotes (called auxospores) attached perpendicular upon the parental cell. Each auxospore develops into an initial cell, which is usually much larger than the parent.

Several cultured strains of Pt already exist in culture collections. Most of these are, however, older than most researchers alive today (De Martino et al. 2011). Cultured strains are notorious for losing wild-type features with time, amongst them it is possible that they have lost also the capacity to have sex. We therefore believe that chances of finding sexual reproduction in Pt are highest with newly established strains.

Where can Pt be found? The species seems to be found predominantly in benthic habitats, in the oval, and rarely in the triradiate shape. Occasionally, cells are swirled up in the water column where they rapidly assume the fusiform shape (Barker 1935). There they probably do not become truly planktonic but reside in the tychopelagos, i.e., drifting in the water layer just above the sediment. Screening of a HTS metabarcode time series of our offshore plankton sample station over the seasons in three consecutive years did not show any evidence of this species' presence there, nor sequences of Pt were found in a HTS metabarcode dataset from surface sediments collected in the Gulf of Naples. Yet the species has been recorded about 10 years ago in a shallow eutrophic benthic community in front of the Stazione Zoologica. This is why we set out to detect the species in this environment over an annual cycle (Plan A). If

successful we planned to bring the established monoclonal cultures together to induce sex under various conditions, and if auxospores obtained, sort out the mating type of the various strains through pairwise crossings. If we fail to find strains this way the back-up plan (B) will be to try to combine strains (<10y) and incubate them under conditions believed to foster sexual reproduction.

Emiliania huxleyi (Eh)

Eh is a unicellular coccolithophore belonging to the eukaryotic Haptophyte division. Coccolithophores are distinguished by their production of calcium carbonate "coccolith" plates. Eh is a major coccolithophore species and regularly forms intense massive blooms in sub-polar and temperate waters. Coccolithophores play an important role in long-term carbon cycling in the oceans and the production of calcareous sediments. Eh has long been used a model for understanding coccolithophore biology and the calcification process. However, the full haplo-diplontic life cycle has not yet been reproduced in the laboratory. This is a potential major bottleneck in the application of genetic approaches. It has long been assumed that Eh exists in two major cell cycle states: diploid calcifying cells and haploid non-calcifying motile cells. There are a number of reports describing the production of motile haploid cells from diploid cultures under a variety of conditions. Moreover viral infection of diploid cells has been shown to promote the transition to haploid motile cells that are resistant to further viral infection. To our knowledge there are no reports of transition of haploid cell cultures to diploid cells. A number of Eh cultures are maintained in the collections of both the Marine Biological Association (Partner 7) and the Station Biologique, Roscoff (Partner 1). The aim of this WP is to develop approaches for cross-fertilization of haploid gametes of Eh leading to the production of diploid cells. Such characterization of the sexual reproductive system has not so far been achieved for coccolithophores. In order to achieve this it is first necessary to fully characterise the motile uniclonal "haploid" cultures that are available in order to enable eventual crossing of mating types.

Ostreococcus tauri (Ot)

O. tauri is a eukaryotic picoalga belonging to Mamiellophyceae, a class which also includes the genera *Bathycoccus* and *Micromona*s. Mamiellophyceae have a worldwide oceanic distribution from polar to temperate and warm oceans. This class is the 4th most represented in term of 18S barcode sequence numbers in the TARA Ocean Cruise. *Ostreococcus* cells which were described as the smallest free living organisms have a simple cellular organization, with single chloroplast and mitochondrion and no cell wall. Thanks to the implementation of genetic transformation tools including gene targeting by homologous recombination, *O. tauri* has become a new model organism for cell biology, ecology, oceanography and for biotechnological applications (Corellou et al., 2009; Lozano et al. 2014).

In our culture conditions *O. tauri* cells are haploid. This feature enables to observe directly a phenotype upon knock out of a target gene. However when the targeted gene is indispensable for cell survival, no recombinants are obtained. Furthermore only one selection gene works efficiently in Ostreococcus (KanMx encodig resistance to G418) making it difficult to modify several loci in one strain.

Sexual breeding would be an alternative to cross lines carrying different genetic markers or transgenes as well as to segregate mutations in cell progenies through classical genetic techniques (Blanc Mathieu et al., 2017).

Under our laboratory conditions, diploid stages of *O. tauri* have not been observed suggesting that upon gamete fusion the diploid phase may be transient. Sequencing of several strains, however, suggests the occurrence of sexual reproduction in *O. tauri* in the sea but experimental evidences are still lacking.

2 Materials and methods

2.1 *Phaeodactylum tricornutum*

Plan A: Shallow benthic communities were sampled fortnightly at sites where the species was found 10 years ago; the upper mm of the sediment was scraped off. Aliquots of such samples were incubated in f/10 medium in Petri dishes and the communities growing on the bottom screened for the presence of Pt under an inverted microscope after one week. This procedure was repeated over an entire annual cycle.

Plan B: Existing cultures (De Martino et al., 2011) were gathered, and crosses were performed as detailed below.

Specified conditions were: small volumes of pairs of exponentially-growing cultures were inoculated in wells of culture plates containing f/10 medium. Three different cell concentrations for each of the two co-cultured strains were tested: about 500, 1000, 2000 cells·ml^{-1}. Six culture wells were also inoculated with a mix of all 10 Pt strains. Culture plates were placed on a shelf under ambient light in a North-facing window, supplemented by cool-white illumination to obtain irradiance of about 80 µmol photons m^2 s^{-1}.

The table illustrates the life form of the strains at the time in which crosses were run.

All strains were crossed with the 'fusiform' Pt10 and the 'oval' Pt3 strain; the same scheme was repeated using different cell concentrations.

Strain code	
Pt1	Fusiform and oval
Pt2	Fusiform (dominant) and oval
Pt3	Oval
Pt4	Oval
Pt5	Fusiform
Pt6	Fusiform
Pt7	Fusiform and oval
Pt9	Fusiform and oval
Pt10	Fusiform
Pt11	Fusiform

Culture plates were visually screened for evidence of sexual reproduction using an inverted microscope.

2.2 *Emiliania huxleyi*

Ploidy levels of selected cultures are being assessed by using either flow cytometry or fluorescence microscopy. For flow cytometry, samples were fixed with 10 µl 0.25% gluteraldehyde solution and 1 µl 10% pluronic acid solution and stained with either Hoechst

or SYBR-green dyes. Samples were then stored at -80°C before processing by flow cytometry.

Selection of uniclonal haploid strains is being attempted by single cell picking, dilution to extinction and sub-culturing techniques (pipetting small volumes from the top of each culture vessel- in theory where the majority of the cells should be haploid and motile).

2.3 *Ostreococcus tauri*

We generated a transgenic line of *O. tauri* RC1110 carrying a H4:KanMX HAPT:Luc transgene using standard transformation protocol (Corellou et al., 2009).

We used also a H4:Nat1 Nitrate reductase knock out line (Lozano et al., 2014).

Both lines were grown to 10 million cells/ml in 10 ml of Artificial Sea Water (ASW) for 0 to 24 hours at 20°C, under blue light (20 µE).

At time 0, both cultures were mixed.

G418 (1 mg/ml) and CloNat (2 mg/ml) were added at either time 0, 12h or 24 h until time 24h.

At time 24 h cells were diluted at 1/10 or 1/100 and transferred in deepwell microplate (1,5 ml per well) in ASW containing cloNat and G418.

Deepwells were incubated for 7 days at 20°C, under blue light (20 µE).

After seven days, growth was detected in 8 wells.

DNA was extracted and amplified by PCR using Nat1 and KanMx specific primers as previously described (Lozano et al. 2014).

3 Results

3.1 *Phaeodactylum tricornutum*

Plan A: Pt was never found in the Petri dishes. Numerous other diatom species, often highly similar to Pt, grew up. Those were isolated into monoclonal cultures and characterized by means of isolating their DNA, PCR amplifying and Sanger sequencing their nuclear encoded LSU rDNA region, and blasting the result against the nucleotide database in NCBI GenBank. However, none of these strains was found to belong to Pt.

Plan B: None of the culture conditions resulted in any visual evidence for sexual reproduction. That is: no auxosporulation was observed.

3.2 *Emiliania huxleyi*

When using flow cytometry, 2 cultures TQ26-1n (RCC1216) and TQ26-2n (RCC1217) from the Roscoff Culture Collection were used as baselines to assess cell ploidy. However, variations in cell florescence between ploidy levels were not immediately apparent. We are now employing quantitative fluorescence microscopy of single cells to a wider range of culture strain types to further characterise ploidy levels.

In experiments to generate stable clonal haploid cultures from the Plymouth Culture Collection, although some cultures were enriched in flagellated cells for periods of time, the cultures would often revert to non-flagellated dominated or mixed cultures. Our observations so far suggest that a clear distinction between haploid motile and diploid non-motile cells is not straightforward. This observation has been brought into sharp focus by a recent publication (Frada et al, 2017) who have demonstrated that the motile flagellated morphotype may be either haploid or diploid and that viral infection of diploid cells may result in a motile life cycle stage that is decoupled from ploidy level (i.e. diploid motile). This is a surprising finding and we are now assessing our results and designing further experiments from this perspective.

3.3 *Ostreococcus tauri*

The very small size of *O. tauri* makes difficult the microscopic observations of sexual reproduction and the identification of optimal conditions for inducing gametogenesis.

We took advantage of the genetic diversity of *O. tauri* (13 sequenced strains) and of the genetic transformation tools available for *O. tauri* to generate strains with selectable phenotypes (i.e resistant to different selection genes):

i) we generated a transgenic line of *O. tauri* RCC1110 carrying a KanMX selection gene under control of the Histone H4 promoter and the firefly luciferase under control of the High affinity phosphate transporter promoter; ii) a RCC745 *O. tauri* knock out line in the Nitrate reductase (i.e. carrying a Nat1 transgene inside the nitrate reductase CDS) was generated earlier (Lozano et al., 2014).

Both lines were incubated for 0 to 24 hr in Artificial Sea Water containing NH_4^+ as a source of nitrogen.
G418 (1 mg/ml) and CloNat (2mg/ml) were added either at Time 0, 12h or 24 h.
After 24 hours cells were diluted 1/10 to 1/100 corresponding to densities between 10 0000 (1/100) and 1 million cells/ml (1/10 dilution).
8 clones resistant to G418 and CloNat were obtained. None of these clones were luminescent.
PCR analysis using specific primers did not allow to amplify the Nat1gene.
This suggests that the 8 lines were false positives resistant to cloNat.

4 Conclusions

The exercise to detect sexual reproduction in the three species was a high risk – potentially high reward effort. Unfortunately, it did not generate the positive results we had aimed for. The reason for trying this in the context of WP7 was that as an alternative to genetic manipulation, selective breeding allows for a natural way of generating strains with favourable combinations of traits.

Regarding Pt, we will continue our efforts to find new strains. In addition, efforts will concentrate on exposing strains to mild turbulence. Their form changes from small-oval to elongate-fusiform when strains transit from benthic to the tychopelagic, which is reminiscent of the tall, elongated F1 cells resulting from sex between usually much smaller parental cells in most pennate diatoms, in e.g., *Pseudo-nitzschia* (e.g., Amato et al. 2007; Levialdi Ghiron et al. 2008). Patil et al. (2015) found genes related to meiosis also in Pt. In addition, Rastogi et al. (2017) uncovered evidence for gene flow among the Pt isolates. This means that sexual reproduction occurs in Pt.

Since diatoms are diplont, i.e., their vegetative stage is diploid, selective breeding permits generating strains homozygous for each of a series of desired traits. And since all these microalgae are small, large numbers of offspring can be kept in multi-well culture plates and screened automatically. This makes selective breeding much easier, cost effective and more rapid that working with larger organisms such as land plants and animals.

The characterisation of ploidy levels and the generation of stable haploid cell cultures remains a goal of the work with Eh. The unexpected complexity of the Eh life cycle and potential for decoupling ploidy level from morphology has added a new dimension to these investigations. Our immediate aim is to develop more sophisticated methods for cell isolation and for assessing DNA content by using fluorescence microscopy.

Regarding *O. tauri*, we have tested so far two lines due to time constrains and our heavy involvement in other Tasks of WP7. These two lines may not correspond to compatible mating types. The approach could be extended to other 11 *O. tauri* strains. We may need also to test a more selective selection gene since cloNat generates false positives.

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