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My project in the King Lab was to develop a method for RNAi-based gene silencing in choanoflagellates, the sister group to animals. RNAi was to be tested on loricate choanoflagellates, which possess an extracellular skeleton (or lorica) made of silicon. The target was to be the recently identified silicon transporter genes. Silencing these genes would prevent silicon uptake and therefore lorica formation, resulting in a phenotype of aloricate (*i.e.* naked) choanoflagellate cells. By quantifying the percentage of loricate and aloricate cells in a culture, and then comparing this to the control cultures, successful gene silencing could be detected.

My first task was to establish cultures of *Diaphanoeca grandis* for the experiments, due to the large lorica and robust growth of these cultures. Fluorescent lorica stains were trialled; an RFP stain was chosen for use after inconsistent results from silica-specific stains. This stain highlights all extracellular components, including the lorica. Counts of loricate and aloricate cells were carried out on multiple slides sampled from the same culture to determine sample variability and confirm the viability of the quantification strategy.

I designed several dsRNA-producing constructs of choanoflagellate silicon transporters for *in vitro* and *in vivo* dsRNA synthesis. The dsRNA produced was encapsulated in an artificial acid-degradable polymer developed by the Murthy Lab (Dept. of Bioengineering, Berkeley), and the resulting microgels fed to *D. grandis* cultures. Preliminary tests were done using fluorescent-labelled DNA; fluorescent food vacuoles were observed indicating that the microgels were ingested. I then tested feeding fluorescent-labelled acid-degradable microgels and after 4 days no fluorescence was seen in the cells, indicating that *D. grandis* could feed on these particles without the degradation killing the cells. Test microgels were made containing control dsRNA. This was recovered intact after *in vitro* digestion, demonstrating that the encapsulation process is viable for use with dsRNA.

Experimental microgels were then prepared using control dsRNA and the silicon transporter targeting dsRNA. *D. grandis* cultures were set up using no microgels (Blank), negative control microgels or target microgels. Small scale preliminary trials found that (a) the percentage of aloricate cells increased in the +microgel cultures compared to the control, (b) the +target dsRNA cultures had the most aloricate cells and (c) cell numbers decreased markedly in the +control dsRNA culture. However when the experiment was repeated using larger volumes and haemocytometer cell counts, I saw that most loricate cells were sticking to the larger microgel particles, forming ‘clumps’ >100 choanoflagellates. The remaining single cells were overwhelmingly aloricate, as the naked cells had a smaller adhesive surface area. The small-scale observations of the first experiment sampled only the free-floating cells and excluded the clumped cells, hence the apparent rise in the percentage of aloricate cells and the low overall cell numbers. This meant that microgels were not a suitable method for developing silicon transporter silencing, as quantification would be determined by the number and size of clumps randomly encountered during sampling. However the *D. grandis* cells appeared healthy and I believe that this method has potential for future transgenics work with phenotypes not related to clumping behaviour.

The second delivery method tested was to feed dsRNA-producing bacteria to the *D. grandis* cultures. Use of a self-lysis plasmid designed by the Anderson Lab (Dept. of Bioengineering, Berkeley) had major problems with PCR amplification and double transformation. Instead I used the dsRNA-producing L4440 plasmid and the

RNase-deficient HT115 *E. coli* strain to deliver dsRNA designed to a ~500bp region of the silicon transporter gene.

Feeding experiments were performed by growing up HT115 in antibiotic-containing liquid medium, pelleting and washing the cells, then adding bacteria to the *D. grandis* culture every 48 hours. Based on growth curve observations cultures were monitored after 2 and 4 days. Cultures were divided into Blank Control (no bacteria added), Negative Control (L4440 only bacteria added) or Target (L4440+silicon transporter bacteria added). Results showed that choanoflagellate cell numbers were higher after bacterial addition, supporting the prediction that *D. grandis* would consume the added bacteria. I found an increase in the percentage of aloricate cells in the +bacteria cultures, however there was no significant difference between the experimental and negative control cultures. Attempts to use qPCR to monitor transcript levels, were hampered by the RNA extracted being mainly bacterial RNA, preventing synthesis of sufficient choanoflagellate cDNA for qPCR.

In subsequent experiments I observed slow bacterial growth rates in liquid media. Further PCRs found that the bacteria lacked the silicon transporter fragment. The HT115 cells were transformed again with the blank and target L4440 plasmids and grown up, as well as an untransformed control. New colony PCRs detected the presence of the L4440+silicon transporter plasmid. These bacteria were grown up and used in further feeding experiments. As before, +bacteria cultures showed a significant rise in the percentage of aloricate cells, however there was no significant difference between the cultures fed with negative control bacteria versus target bacteria.

Unexpectedly, the untransformed bacteria grew on the LB-Ampicillin-Tetracycline plates. In parallel with the feeding experiments, the bacteria were used for sequencing. The sequencing found that all three strains contained blank L4440 plasmid sequence, meaning that the original bacterial stocks contained the L4440 plasmid, and that the target bacteria in fact contained two plasmids. These double-transformed cells were presumably able to grow on the plates by being spatially removed from the single-plasmid cells. However in liquid media, and in the choanoflagellate cultures, the selective pressure would favour the loss of the L4440+silicon transporter plasmid and the domination of the L4440 only cells. This was confirmed by the inability to detect the silicon transporter sequence from bacteria grown in liquid LB+Ampicillin+Tetracycline. Therefore the feeding experiments in effect only involved the negative control bacteria, explaining the absence of any significant effect for gene silencing. The discovery of this problem came too late for me to repeat the experiments in the Berkeley, however using further RNAi experiments are being performed in Cambridge, using new HT115 *E. coli* free from any prior L4440 transformation and the quantification methods developed in the King Lab.