Searching for R-bodies in Trunk River

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Abstract

R-bodies are a still largely unexplored proteins able to induce a killer trait in bacteria and their associated symbiont hosts. This study examined two topics: Searching for the presence of R-bodies in Trunk River, MA, USA and the prospect of feeding ciliates *E.coli* containing active R-bodies. Primers were designed based on aligned sequences from the IMG database and PCR was conducted on four DNA extractions from Trunk River: Water column, sand, deep sediment and lemonade. No PCR bands were acquired. Ciliates were fed fluorescently labeled *E.coli* cells containing either truncated or active R-bodies and their response was evaluated using fluorescence microscopy. Cells were observed to explode when digesting cells containing active R-bodies. An overall difference of 25466 ciliates/ml was observed between the two treatments evidencing a negative impact on ciliate populations when exposed to active R-bodies.

Introduction

R-bodies are insoluble ribbon like proteins originally found in the ciliate endosymbiont Caedibacter taeniospirals (Pond et al. 1989). These proteins have two distinct states: folded or stretched. When folded the proteins are small cylinders and by stretching they turn long and flat, almost resembling confetti. Five different R-body morphology types have been described so far with R body type 51 being the best described (Figure 1). The trigger for the stretching mechanism of R-body varies between the morphology. Type 51 R-bodies respond to pH changes, while type 7, Pt and Pa trigger by heat. This change in structure has been shown to be reversible in Type 51 R-bodies by repeatedly changing the pH (Polka and Silver, 2016). Homologs of the Reb genes coding for the R-bodies are almost exclusively found within the Proteobacteria (Raymann et al. 2013). The MBL sampling site Trunk River is a brackish sulfidic pond rich in Proteobacteria, leading to the first main hypothesis of this study: Are there R-bodies in trunk river?

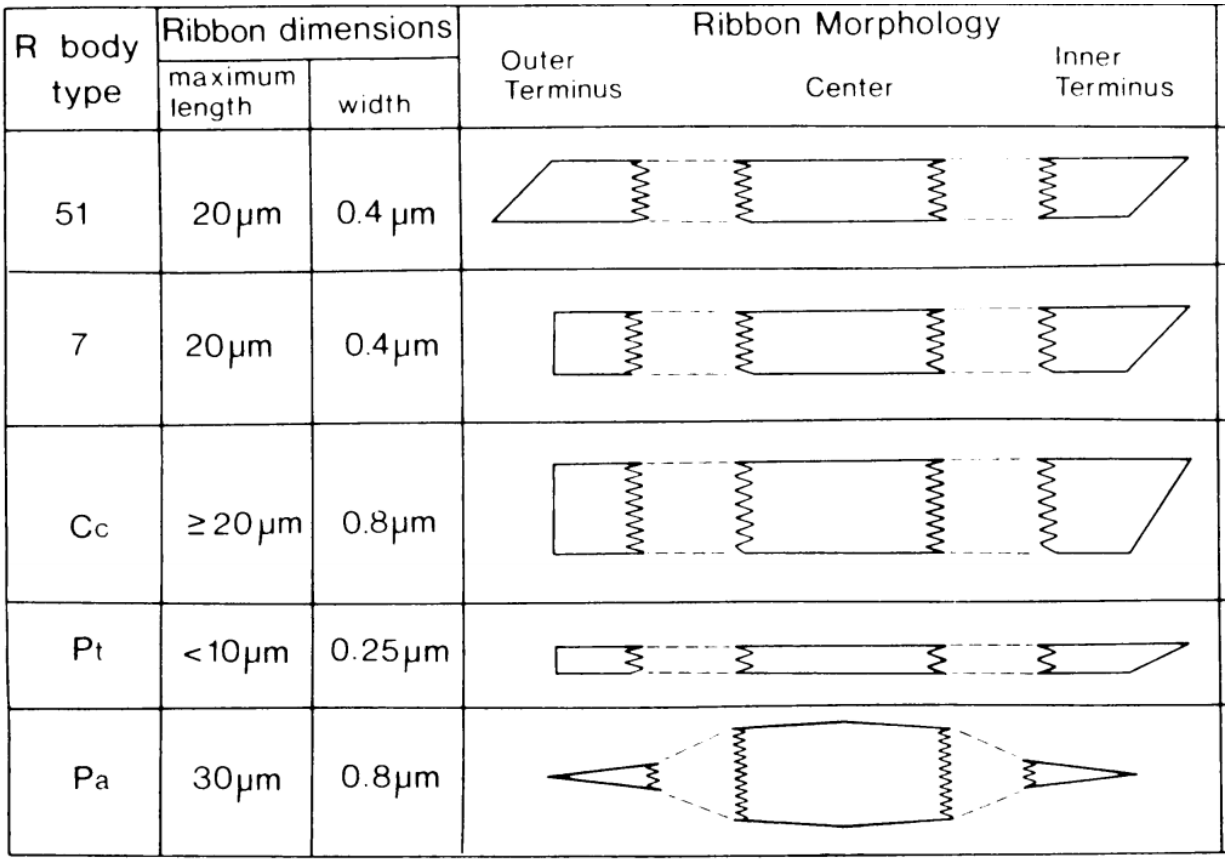

Figure 1: Five R-body morphologies, (Pond et al. 1989).

With regards to their ecological importance, R-bodies are a natural toxin delivery system between ciliates as seen on figure 2 (Sonneborn et al. 1938) (Dippel 1958) (Mueller 1965). The killer strain of paramecium hosts R-body expressing bacteria. These endosymbionts are shed to the surrounding environment. A sensitive paramecium strain will feed on this bacterium by capsuling it in a food vacuole. As the bacterium is digested, the pH decreases triggering the R-body to extent. When this happens, the food vacuole is pierced and the toxins are released into the cytoplasm of the paramecium, killing the ciliate.

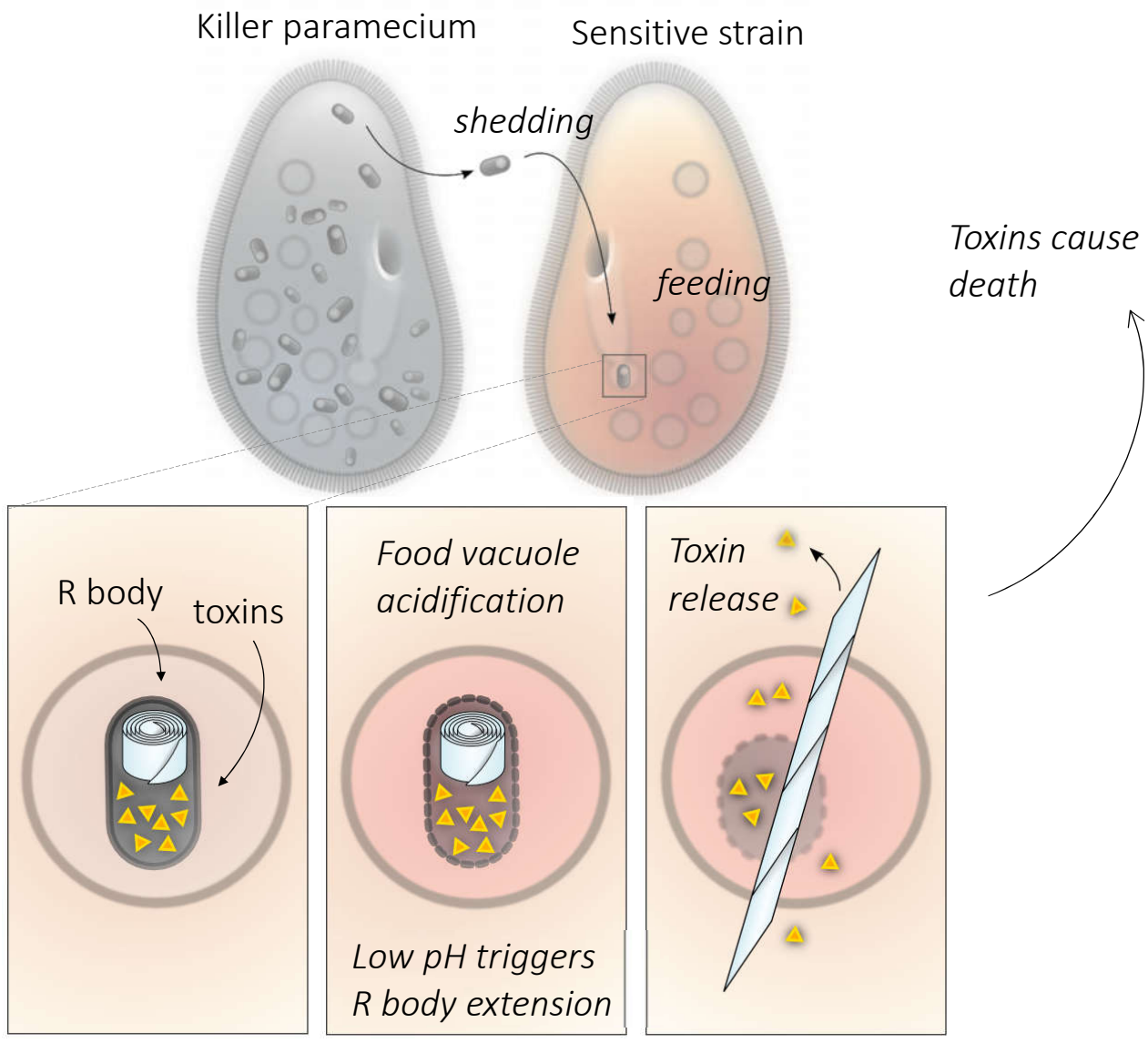

Figure 2: Killer trait mechanism of type 51 R-bodies.

However, the toxins associated with R-bodies have never been described and evidence suggests that the R-bodies alone can lyse spheroplasts (Polka, unpublished). This leads to the second main hypothesis of this study: Are the extension of R-bodies alone enough to kill their sensitive hosts?

To test both of these hypotheses primers were designed based on RebB homologs and PCR was performed on DNA extracted from Trunk River pond. Furthermore, unclassified ciliates where fed *E.coli* containing both active and truncated R-bodies to elucidate whether the R-bodies alone are sufficient to kill ciliates.

Methods

BLAST

Reb homologs were found by BLASTing against the entire IMG isolate and metagenome database. The RebB protein from the Caedibacter taeniospiralis plasmid was used as reference sequence as it is responsible . 900 protein sequences from isolates were retrieved, 500 from isolates and 400 from metagenomes. The retrieved metagenomics sequences spanned highly variable environments including Antarctica, upper troposphere, soda lakes, oxygen minimum zones and hydrothermal vents. Many of the retrieved amino acid sequences were annotated simply as killer trait.

The reference RebB sequence

>Caedibacter_641212162 YP_025468 RebB [Caedibacter taeniospiralis plasmid pKAP298: NC_005915]
MSNVNSQITDSVTQTNTKILGEMPAFTTGSLMQMATQAAGLSIQNSVTNQQQSNMLHQASTTQGMSILYSV
DTAANAQAIGSVNRSNDTSRLTDALAVIKAAKNG

Primer design

The retrieved RebB homologs were aligned in Jalview using the Muscle algorithm and coloured with clustalx seen in figure 3. Highly conserved areas were chosen for primer design and the final primers were as follows:

RevBF: 5 GARATHATNGAYGCNGTNATNG 3

RevBR: 5 GTNAGNAGNGSNGTNAGRTTRTCNGC 3

Figure 3: Muscle alignment of RebB homologs retrived from the IMG database. Amino acids marked in red were used to design the forward primer and blue were used to design a reverse primer.

Sampling site

Four samples were collected from Trunk River pond in falcon tubes. Different microbial niches were sampled: Water column, upper sandy layers, deeper sediment layers full of methane as seen from bubbles and yellow water termed “lemonade”.

DNA extraction

DNA was extracted using the PowerFecal kit as described per manufacturer instructions. For sediment samples 0,5 grams were used. For liquid samples 250 µl was used. DNA concentration was measured by Quantus fluorometer as seen in table 1. The lemonade extraction was below detection limit.

Sample	Concentration [ng/µl]
Water column	1,44
Lemonade	<Blank
Sand layer	9,1
Sediment	18

Table 1: DNA concentrations from the four extractions.

Polymerase chain reaction

A mixture with a total volume of 50 µl was prepared for each PCR as outlined in table 2. Each DNA extraction was tested in quadruplicates. Different ratios of primer to DNA template was tested (1:2, 1:3, 1:4). The PCR program ran for 27-31 circles and consisted of a gradient from 40°C-65°C.

Reagent	Amount [µl]	Reagent	Amount [µl]
		dH_2O	30,5
Tag Master Mix	25	10x Buffer	5
		dNTP	8
Forward primer	1-3	Forward primer	2
Reverse primer	1-3	Reverse primer	2
DNA	2-4	DNA	2
dH_2O	18	LA Taq	0,5
Total	50	Total	50

Table 2: PCR recipes. Values in µl.

The PCR product was evaluated by gel electrophoresis on a 1 % agarose gel at 100 mV.

Experimental setup for ciliate explosions

In this experiment fluorescently red *E.coli* cells had been engineered to express green fluorescently labeled R-bodies making for easy identification of both *E.coli* cells and R-bodies. For the actual setup, 3ml of a ciliate culture growing on a biofilm was transferred to small petri dishes. The ciliates were grown under three different conditions:

1. *E.coli* containing active R-bodies
2. *E.coli* containing truncated, inactive R-bodies
3. Ciliates without anything added functioning as a control

10 µl of either kind of *E.coli* was added to petri dish 1 and 2 and mixed using the pipette tip. All three petri dishes were incubated at room temperature under tinfoil to protect the fluorescently labeled cells from bleaching. For evaluation, 3 µl was extracted from each petri dish, transferred to a slide and imaged using microscopy. Phase-contrast was used to locate the ciliates. Fluorescence microscopy was used to evaluate whether or not the ciliates consume the *E.coli* cells. The

microscope used was a Zeiss Axio scope connected to a computer for snapping imaging and videos.

Cell counting

50ml samples were taken from each ciliate conditions and fixed in 4% formaldehyde. Cells were quantified using a haemocymeter. Five times fifteen squares were counted and the total concentration of ciliates was calculated by the following equation:

$$\frac{Ciliate\ count \quad 4000}{number\ of\ squares\ counted} = ciliates/ml$$

Results and discussion

The presence of R bodies in Trunk River

After 11 different PCR reactions with primers designed to target R-bodies no bands were observed. At DNA template concentrations of 4 µl a slight smear appeared (figure 4) likely due to the high amount of DNA in the mixture and not signifying an actual band. The approach was ultimately abandoned in favor of microscopy.

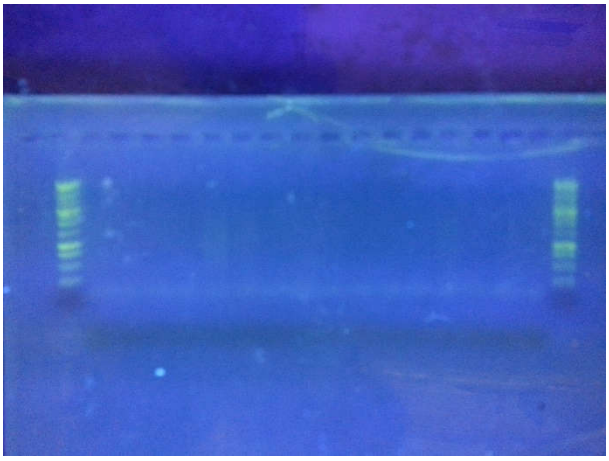

Figure 4: Picture of a gel with 4x4 samples. The left and rightmost lanes are DNA ladders. No bands were present

Feeding ciliates active R-bodies

Figure 5 shows pictures of ciliates before and after addition of *E.coli* cells. The red and green fluorescence in the shape of cells observed within the ciliates after addition of the *E.coli* evidences the successful uptake of these.

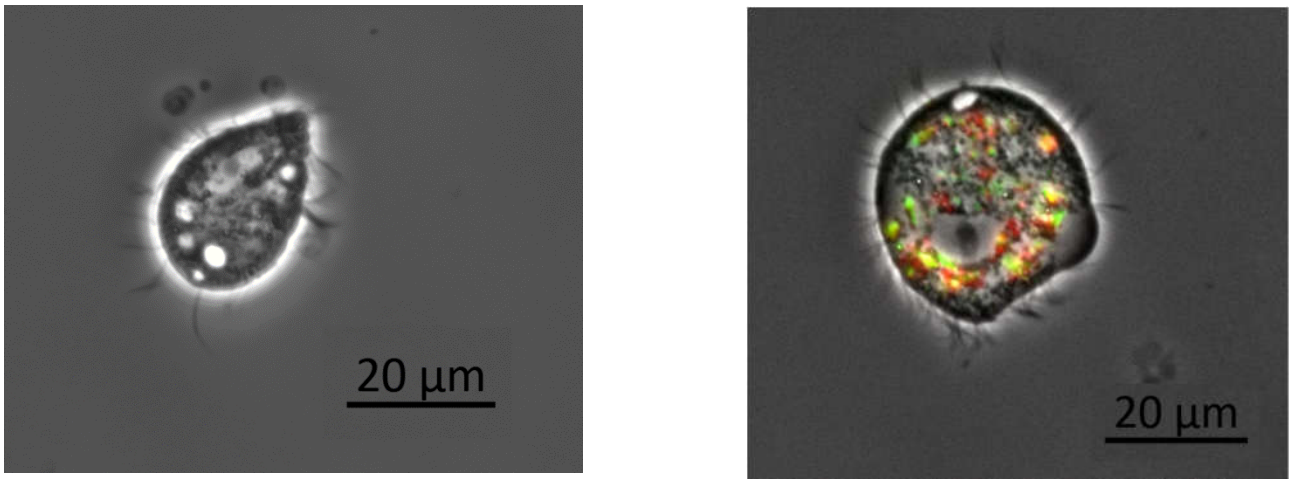

Figure 5: Phase contrast microscopy at 40x of a ciliate before addition of E.coli (Left). A layered phase contrast and fluorescence microscopy of a ciliate containing E.coli cells (Right).

After instruction of the active R-bodies, the cell membranes became leaky, causing an expansion in cell volume from water uptake due to the osmotic pressure within the cell (figure 6, left). Once the ciliate reaches critical volume the membrane will rupture, exposing the cytosol to the environment (figure 6, right). The dead ciliate is still fluorescent, indicating intact R-bodies.

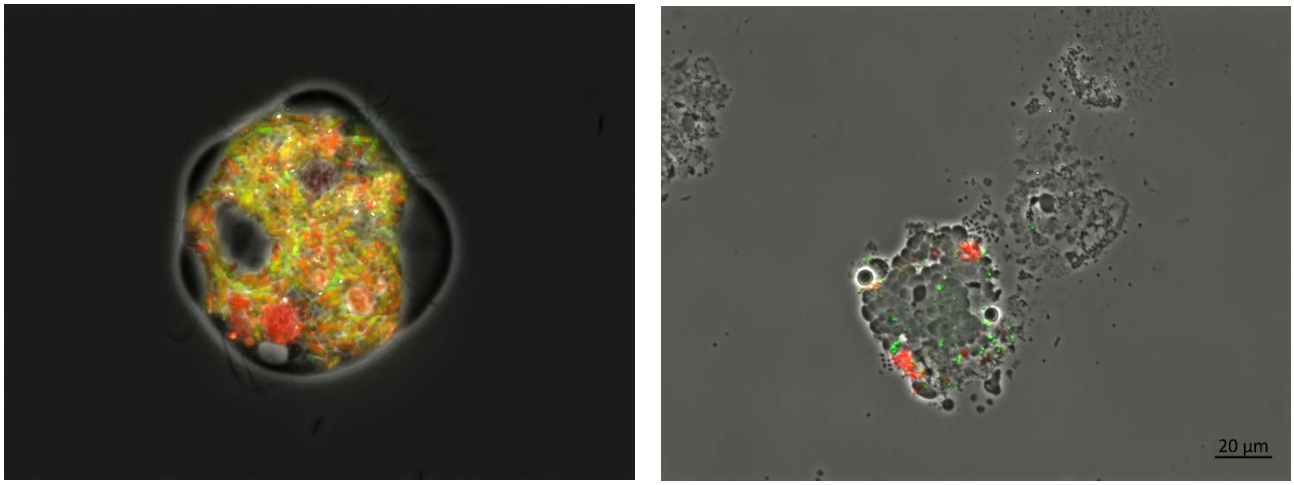

Figure 6: An exploding ciliate (left) and the remains after explosion (right).

Figure 7 shows the ciliate concentrations under three different growth conditions. At time point 0, there is near identical concentrations in all three samples ranging from 2666 ciliates/ml in the control to 4533 ciliates/ml in the truncated R bodies. After 22 hours the concentrations are different. The control and truncated sample has majorly increased to 12933 ciliates/ml and 30400 ciliates/ml respectively while the active R-body sample has seen a slight increase to 4933 ciliates/ml. The increase in ciliate count in the control is explained by growth due to consumption of the biofilm in the medium. Likewise, the increase in the truncated sample is caused by both the consumption of the already occurring biofilm as well as the introduced *E.coli* cells. The difference in ciliate concentration between the sample with truncated R-bodies and active R-bodies is major at 25466 ciliates/ml. This difference suggests, that the consumption of *E.coli* cells containing active R-bodies does negatively impact the ciliate population, aligning perfectly with the microscopy observations of exploding ciliates.

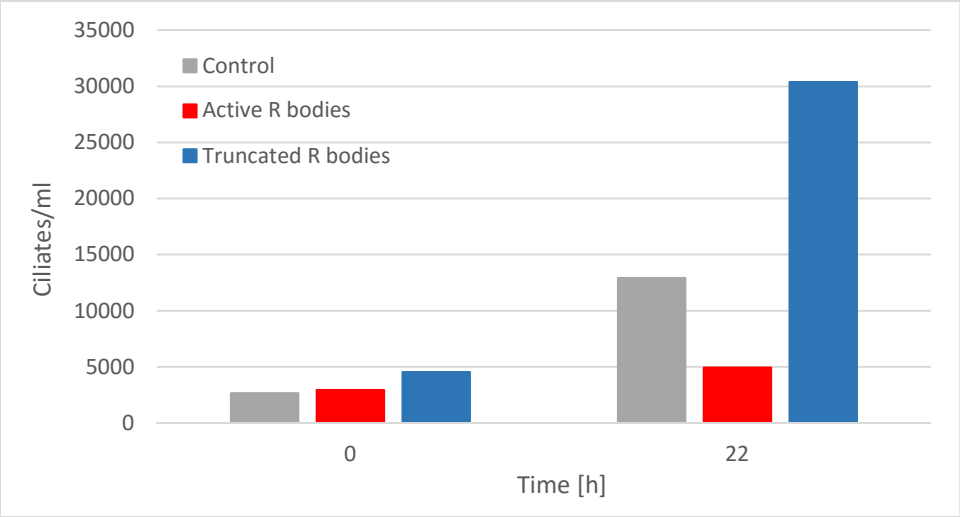

Figure 7: Ciliate concentrations growing under three different conditions: E.coli containing active R-bodies (red), E.coli containing truncated R-bodies (blue) and a control growing on a bacterial biofilm (grey).

One question still remains: If the R-bodies do indeed make the ciliates explode, why do we observe a small increase in the ciliate population feeding on *E.coli* containing active R-bodies? The answer can be found in the ciliates ability to form cysts (Figure 8). Flourescence is observed within the cysts in the samples containing truncated R-bodies, suggesting that the cysts originate from the ciliates eating *E.coli*. In the sample with active R-bodies no cysts are found containing fluorescence, while *E.coli* cells and R-bodies can been seen around the cysts. These cysts inflate the ciliate count after 22 hours of incubation. It could be hypothesized that the ciliates in the sample with active R-bodies use the cysts as a way to combat the R-bodies by lying dormant until the R-bodies disappear.

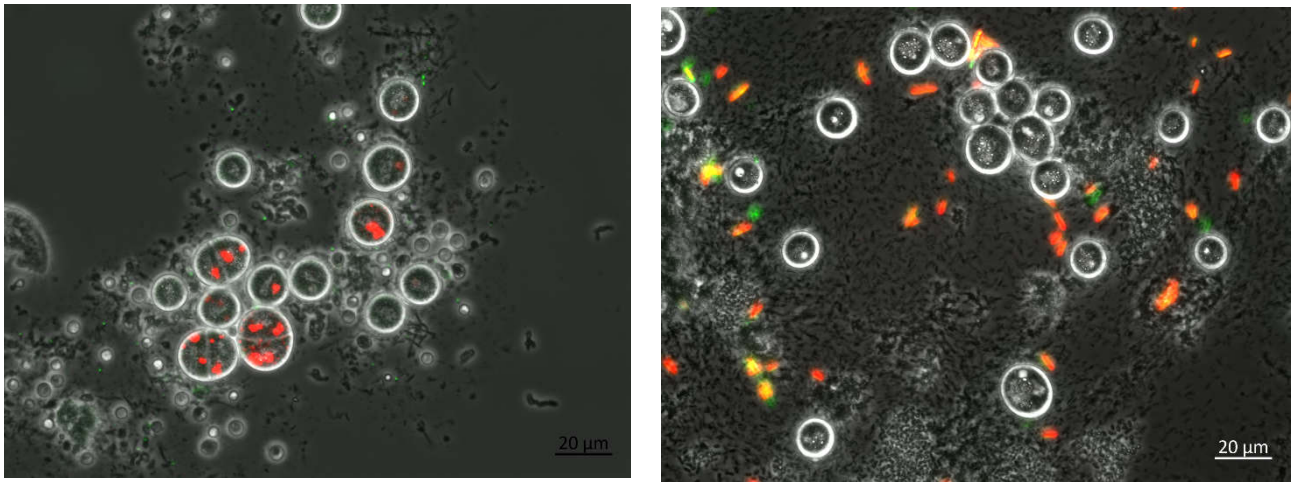

Figure 8: Pictures of ciliate cysts at 10x magnification. Cysts containing E.coli cells and truncated R-bodies (Left). Cysts with no intracellular E.coli

Future perspectives

The failure in getting a clear PCR band should be addressed, as the R-body homologs appear to be widely spread as evidenced by the BLAST search. The forward primer appears highly conserved, making for a good primer, while the area for the reverse primer is less conserved. Indeed, the BLAST results from the metagenomes vary and a new reverse primer should be designed by aligning only these sequences clear of any isolate homologs.

The ciliate used in the study should be sequenced. Quantification of live/dead ciliates should be improved by staining and a more reliable way of counting. Cysts greatly influence the ciliate count when the population is in low numbers and this needs to be addressed in a better way for more convincing comparisons.

Doing electron microscopy on both life and dead ciliates would help elucidate exactly if/how the R-bodies pierce the ciliate membranes. For this the ciliates would need to be stable and therefore fixed in a way that makes them immobile without destroying the sample.

It would be interesting to do a study on the ciliate cysts with a focus on their germination. How can germination be induced and would this be different in the presence of active R-bodies?

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For any videos of the ciliates exploding, please e-mail me and I would be happy to share them.

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