Ultra-small cells and a new isolate of the Marinilabiliaceae and a charismatic methanogen - accidents and discoveries from Trunk River, Woods Hole

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I. Abstract

Ultra-small bacterial/archaeal cells have a diameter below 0.2um and often show streamlined genomes, pointing at a symbiotic or free-living life style. During the Microbial Diversity course in 2015 a Parcubacteria genome was assembled from the Trunk River Lemonade, and 16S rRNA amplicons showed evidence of small bacteria in this environment. The aim of the mini-project was to explore if ultra-small cells are indeed abundant in the Lemonade, to make these small cells visible and to track them down by cultivation approaches. Fluorescence (FISH) and electron microscopy appeared suitable to see ultra-small cells, although cells could not be distinguished unambiguously at all times, as they are at the limits of resolution of scanning electron microscopy and also fluorescence microscopy. Cultivation attempts yielded one isolate, a long flexible and very thin rod, obligate anaerobe, which belongs to the family of Marinilabiliaceae, phylum Bacteroidetes, representing at least a new species. As a side project a Methanosarcina enrichment, was investigated due to its charismatic and curious fluorescent dots of F420, which are usually not observed in Methanosarcina.

II. Introduction

Usually a filter with a pore size of 0.2um is used in environmental microbiological studies to harvest bacterial cells from the environment for further analysis. Despite that, there exist bacteria and archaea that pass through this pore size, and have a diameter below 0.2um. They are referred to as ultra-small bacteria/archaeal cells, but also nanocells, dwarf cells, midget cells, nanosized cells are terms used to describe them (Duda et al., 2012; Luef et al., 2015). Bacteria of this size are generally thought to have a small genome size, and some are missing essential pathways (Nelson and Stegen, 2015) and have genomes, where large parts remain unannotated (Nelson and Stegen, 2015). Small genome size and missing pathways hint at a symbiotic life style (Nelson and Stegen, 2015), but also free-living organisms have been found to be very small, like Actinobacteria (Hahn et al., 2003), with streamlined genomes. The small free-living cells are usually thought to occur under oligotrophic conditions.

During the Microbial Diversity course 2015 a metagenome of the Lemonade at Trunk River pond, which forms after digging a hole into the decaying plant material layer, was done.
Additionally, 16S rRNA sequencing of the lemonade following its succession over time at different depths was carried out. From this metagenome a full genome of an OD1 (Parcubacteria) was obtained, which is part of the candidate phyla (Hug et al., 2016). Furthermore, 16S rRNA amplicon sequences showed that members of the Microbacteriaceae, which are known to be small, were present and accounted for up to 4% of all reads.

The small size of ultra-small cells might not only be an adaptation to oligotrophy as members were isolated from oligotrophic to eutrophic lakes in Europe and Asia (Hahn et al., 2003). To be small might also be an adaptation against predation and the surface to volume ratio increases, which may make ultra-small cells more competitive for nutrients. Generally, ultra-small cells occur in many phylogenetic groups and is of polyphyletic origin. Regarding the evidence of small cells in Trunk River we asked the question if ultra-small cells are abundant in Trunk River Lemonade, despite the nutrient rich environment and if we can detect ultra-small bacterial/archaeal cells using microscopy and cultivation. Regarding their supposedly reduced genome, media supporting heterotrophs were chosen.

As a side project an enrichment culture, bubbling with methane (Figure 11), was investigated further, because unusual dots of F420 in the enriched methanogen were observed using fluorescence microscopy (Figure 12) (Doddema and Vogels, 1978).

III. Methods

A. Sampling site
Samples were taken at Trunk River, which is a freshwater/brackish basin containing a lot of organic material with seawater intrusions and important sulfide and methane cycling. For the ultra-small cell mini-project samples from the lemonade at Trunk River Pond (see introduction) were taken.

B. Cultivation

1. Ultra-small bacteria
Seawater complete (SWC, see supplementary material) and 5YE agar (per liter of ddH2O 5 g yeast extract and 15 g Agar) plates were used as a substrate to grow ultra-small cells. 100ul of sterile filtered (0.2um pore size) water from Trunk River lemonade were spread on plates and then incubated either oxic, or anoxic in an anaerobic jar or in the anaerobic glove box, all at 30°C. Plates were inspected every day for growth of colonies. The isolate related to Alkaliflexus was grown anaerobically on SWC, but streaking was done aerobically.

2. Methanogens
The methanogens were accidentally grown in a Pfennig bottle containing freshwater acetate and methanol medium incubated at 660nm aiming to enrich phototrophic nonsulfur bacteria (see supplementary material for medium). Intensive bubbling indicated gas production in the bottle. Transfers of the cultures were also grown in this medium (see supplementary
material) and grown under 660nm. Sand from the river bank of Trunk River was used as an inoculum.

C. Fluorescence microscopy DAPI and Sybr gold staining
Water samples from Trunk River were fixed with formaldehyde to a final concentration of 3.7%, because of high organic content a higher percentage was chosen. It is critical to fix and filter the sample the same day and to add 10ml of PBS before adding 1.5ml of sample to the filter tower. The 0.02um pore size Anodisc or 0.025um VSWP filter were sandwiched between a supporting filter (0.45um pore size) beneath and a 0.2um GTTP02500 filter (Millipore), to remove big cells. For methanogens only the 0.2um GTTP filter was used. Filters were air dried, placed on a drop of 1ug/ml DAPI solution and incubated for 3min. Filters were dried on a piece of Whatman paper and then mounted with Citifluor/Vectashield 4:1.

For SYBR gold staining the same filtration procedure was used. The filter was placed upside down on a drop of 200x SYBR gold solution incubated for 15min in the dark and mounted 5:1 with glycerol and 0.1g/ml ascorbic acid in PBS as an antifade solution.

D. Mono-FISH
Samples were fixed and filtrated as described before. 25mm filters were cut into one-eighth and labelled with a pencil. The pieces were dipped into 0.1% agarose and dried at 37°C. 18ul of hybridization buffer were mixed with 2ul of probe. The filter sections were placed on a droplet of this mix on a slide (facing down). The slide was placed into a 50ml Greiner tube and incubated at 46°C for about 2 hours. Filters were transferred to the washing buffer for 25 min at 48°C and then rinsed with ultrapure water and dried on a Whatman paper. The filters were counterstained with DAPI and mounted with Citifluor/Vectashield as described previously. The following probes were used: EUB338I-III, ARCH915, and a NON probe (Table 1). A probe for Parcubacteria was designed by Dimitri Meier, based on the metagenome obtained from 2015, DOPE-FISH was used for labelling the probe (Stoecker et al., 2010).

Table 1 FISH probes used to detect ultra-small cells.

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<th>FISH type</th>
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<td>Parcubacteria, Lemonade 2015</td>
<td>GGATAACGCTTGAGGTCTCT</td>
<td>20</td>
<td>2x Cy5</td>
<td>DOPE</td>
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<td>Bacteria</td>
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<td>4xAtto488</td>
<td>MiL</td>
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<tr>
<td>ARCH915</td>
<td>Archaea</td>
<td>GRGCTCCCCCGCAAATTCTT</td>
<td>35</td>
<td>Cy3,Cy5, Atto388</td>
<td>DOPE+MiL</td>
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<tr>
<td>NON288</td>
<td>Nonsense</td>
<td>ATCTACGGGAGGCAGC</td>
<td>35</td>
<td>4xAtto488</td>
<td>MiL</td>
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</table>
E. Scanning electron microscopy (SEM)
Samples were fixed and filtered as described earlier. For SEM the filter pieces were subsequently dried in increasing concentrations of ethanol 15%, 30%, 50%, 75%, 100% (15min each), then they were critical point dried in the microscopy department in Lilly and sputtered with 10nm of platinum. Samples were inspected at the Environmental SEM of the course and the SEM of the Microscopy Department at Lilly.

F. Bacterial and archaeal 16S rRNA gene sequencing
DNA extraction from the microcolonies was done by boiling cells of one colony for 5min in 10ul of alkaline PEG200 (60g PEG200, Sigma, with 0.93ml 2M KOH and 39ml water, pH of about 13.4). 2ul DNA were used in a 25ul PCR reaction containing 12.5ul Promega GoTaq Master Mix, the 16S_8F and 16S_1391R bacterial primers (2ul each) and 6.5ul water were run on a thermocycler with an initial denaturation of 2min at 95°C, 30sec of denaturation at 95°C, annealing at 55°C for 30sec, extension 72°C for 1.5min and a final extension after 30 cycles to obtain a near full-length 16S rRNA gene sequence, which was sent for Sanger sequencing.

To sequence the 16S rRNA gene of the methanogen enrichment, 2ml of the enrichment were pelleted, the DNA from the pellet was extracted with the Maxwell Blood extraction kit. Two archaeal forward primers were tested (4FA, 333FA) combined with the 1100RA primer, respectively. A gradient PCR was run at 55, 52, 50 and 48 °C and 55°C were found to be the best annealing temperature for both primer pairs, so the same protocol as for the bacterial 16S rRNA gene PCR could be used (Figure 14). The products of both primer pairs were sent for Sanger sequencing.

Important to note is that the sequences obtained were not from a pure culture, as a lot of bacteria were also present in the enrichment, but the selectivity of the primer set towards Archaea made it possible to obtain the 16S rRNA gene sequence without having a pure culture. This method could also be used for other enrichments, where some taxonomic information is available, in this case from microscopy and methane production, but could also come from e.g. FISH or pigment analysis. With this method it is possible to get sequence information before obtaining a pure culture, which is difficult for some organisms and takes a lot of time in some cases, or might be even impossible.

IV. Results & Discussion

A. Microscopy of ultra-small cells
Ultra-small cells are usually thought to live symbiotic or in oligotrophic environments. In 2015 a genome of a Parcubacterium (OD1) was obtained from the “Lemonade” from Trunk River. Additionally, 16S rRNA data contained sequences of Microbacteriaceae, which are known to be small. They seem to be abundant in the lemonade, which is not an oligotrophic environment. Different methods were tested in order to make ultra-small cells visible, and to identify them as cells. In Figure 1 SYBR gold was used, which is a very bright stain. The faint
very small dots are supposed to be viruses, also called virus-like particles (VLP), whereas the brighter spots should represent bacteria or archaea.

While this is a great method to make viruses and bacteria visible, it is impossible to unambiguously discriminate between very small cells and viruses or other stained particles. Nevertheless, only 1.5ml of the lemonade sample were filtered (with a 0.2um prefiltration) and it is clearly visible that ultra-small cells and viruses are very abundant in the sample. Illumina sequencing of 16S rRNA amplicons and metagenomics of the lemonade during the MicrobialDiversity course 2015 already revealed that there should be small cells present.

![Figure 1 Lemonade, <0.2um fraction on a 0.02um Anodisc filter, SYBR gold staining 100x objective](image)

Also DAPI-staining was tested on Anodisc and VSWP filters, the VSWP filters are not as flat as the Anodisc filter, which makes it harder to have all cells/VLPs in one plane (Figure 2).

![Figure 2 Lemonade, <0.2um fraction on an I) Anodisc filter, pore size 0.02um II) VSWP filter, pore size 0.025um, 100x objective](image)
Mono-FISH with EUB revealed some cells, but in the NON probe filters also some “cells” were stained (Figure 3). It seems that the Anodisc as well as the VSWP filter are not fully compatible with the FISH and CARD-FISH protocols, often a very bright background fluorescence has been observed. The FISH and CARD-FISH protocol would have to be adapted to these filters, or different filters could be used. To avoid too many virus-like particles and focus more on cells a 0.1um filter instead of a 0.02um filter could be use, which might also be more compatible to FISH and CARD-FISH. No signals of the newly designed Parcubacteria-probe were found. In 2015 they were found in a metagenome of late successional lemonade from Trunk River, this year only early lemonade could be sampled, due to rain and storm events.

![Figure 3 Lemonade, <0.2um on a 0.02um Anodisc filter, DAPI + FISH signal (EUB) overlaid](image)

Signs of archaea in the small fraction were found with FISH (Figure 4). Some archaea are known to be very small. In future the archaeal primer sets could be used to amplify the 16S rRNA gene combined with Illumina amplicon sequencing of the <0.2um fraction of the lemonade, which could confirm these findings and show the importance and occurrence of small archaea in this environment. Also a metagenome was done of the small fraction, which could be analyzed with a focus on archaea, I could not analyze these data, because they came back only one day before the end of the course. These data might also contain viral DNA.
Figure 4 Lemonade, <0.2μm on a 0.02μm Anodisc filter, DAPI + FISH (Archaea) overlay

To make ultra-small cells visible I applied scanning electron microscopy at the Central Microscopy Facility at MBL. With SEM it was possible to see some ultra-small or thin cells (Figure 5). To unambiguously identify ultra-small cells, transmission electron microscopy should be used (Luef et al., 2015), as with SEM it is hard to distinguish small particles from small cells as SEM is at its resolution limits at these high magnifications.

Figure 5 Lemonade, <0.2μm on a 0.02μm Anodisc filter, SEM, platinum sputtered

B. Cultivation of ultra-small cells

After one week of incubating sterile filtered sample from the Lemonade on a SWC plate in an anaerobic jar a single colony on one of the plates was detected. The colony was streaked out, and after another week tiny colonies were visible under the dissecting microscope,
which also did not grow much bigger when incubating longer. The microcolonies, barely visible by eye without microscope were below 100um in diameter (Figure 6).

Figure 6 Axio zoom, microcolonies, SWC plate, anaerobic jar grown, isolated from sterile filtered Lemonade

Looking at a colony using a wet mount of squashed agar revealed long straight cells with a very thin diameter (app. 0.2-0.4um) (Figure 7). In liquid SWC no movement could be observed, cells on a wet mount were fragile and membrane disintegration started quickly. Looking at a squashed agar piece with a colony on top uncovered their motility, which is probably gliding motility (Figure 8), also the cells looked much longer and more curved.
Figure 7 Liquid part of squashed agar with colony of isolate, phase contrast 100x, microcolony

Figure 8 Squashed agar with colony of isolate, phase contrast 100x, microcolony
To test if this culture is anaerobic, it was incubated under aerobic conditions, in an anaerobic jar, and in an anaerobic chamber. Growth of colonies was only observed under anaerobic conditions, and not on the aerobic plate. Plating was done under aerobic conditions, so it seems the organism is not sensitive to short exposure to air during streaking of the culture.

Sequencing of the 16S rRNA gene showed that the organism belongs to the phylum Bacteroidetes and is related to the genera Alkaliflexus and Mangroviflexus according to a NCBI blast search. According to The Prokaryotes (Rosenberg, 2014) the organism belongs to the family of Marinilabiliaceae (also referred to as Marinilabiaceae), all members are obligatory anaerobic and grow as long and flexible rod-shaped cells, which is in accordance with our findings. The consensus sequence of two separate PCR products sequenced showed that it is 96% similar to an Alkaliflexus sp. isolate and 95% similar to Mangroviflexus xiamenensis and also 95% similar to Alkaliflexus imshenetskii. The low similarity to other isolated species means that the new isolates definitely represents a new species, but might even represent a new genus within Marinilabiliaceae.

Figure 9 The tree was obtained from Prokaryotes (Rosenberg, 2014). Phylogenetic reconstruction of the family Marinilabiliaceae based on 16S rRNA an created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. Scale bar indicates estimated sequence divergence(Rosenberg, 2014).
C. Methanogens (unrelated to ultra-small cells) – side project

During the enrichment for non-sulfur phototrophs at 660 nm one bottle started bubbling instead of growing phototrophs (Figure 11) a video of the bubbling bottle was put on youtube (https://www.youtube.com/watch?v=VDVdBxAn3xY).

Figure 11 Enrichment culture containing methanogens with methane bubbles “champagne”

Under the microscope beautiful Methanosarcina-like aggregates were observed (Figure 12). On the right side F420 autofluorescence of methanogens was used to confirm they are methanogens. Interestingly spots of concentrated F420 fluorescence were observed, which are usually not seen in methanogens. To explore if these dots are visible on the surface of the methanogen I did SEM (Figure 13) with the environmental SEM (left) and the SEM (right) at the Central Microscopy Facility at MBL. The pictures do not reveal structures on the
surface of the methanogen. GC was used to confirm the bubbling gas contains methane (data not shown).

![Image](image_url)

Figure 12 Methanogen enrichment, wet mount, left: phase contrast 100x right: F420 autofluorescence, alexa filter

![Image](image_url)

Figure 13 Methanosarcina enrichment on a GTTP filter, critical point dried and sputtered, I) environmental SEM, II) SEM at Lilly

This methanogen is fragile and starts to disrupt when put under a slide. The enrichment was transferred twice, but bacteria were still abundant in the culture. Therefore, specific archaeal primers were used to amplify to 16S rRNA gene in order to do Sanger sequencing. Blasting of the obtained archaeal sequence revealed the organism belongs to Methanosarcina, with 99% sequence similarity to *Methanosarcina vacuolata*. A metagenome of the culture was done and the full methane pathways could be found (analysis: Grayson Chadwick). It would be very interesting to map the metagenome of the enrichment to the full genome of *Methanosarcina vacuolata* to see if there are differences in parts of the genome, which could explain formation of dots. It has to be said that these dots
are not always present, but often. Bottles were incubated at 660nm, it would be interesting if this light source has an influence on the fluorescent dot formation.

Figure 14 Results of the gradient PCR of the Methanosarcina enrichment Primer sets were tested: 4FA+1100RA (4FA) and 333FA – 1100RA (333FA), 1% agarose gel, 100V

V. Conclusions

- Ultra-small cells do occur in nutrient-rich environments like the Lemonade at Trunk River
- It is challenging to visualize ultra-small cells unambiguously, TEM would be another option, or CARD-FISH on 0.1 um filters
- Archaea might be an important fraction of the ultra-small cells, which could be further investigated by archaeal 16S rRNA of the <0.2um fraction + Illumina sequencing, also a metagenome of the small Lemonade fraction was done but could not be analyzed, as sequences arrived two days before leaving, but is available for further analysis, and might contain viral DNA too
- Some cells are passing through the sterile filter – should be kept in mind if 100% sterile filtration is required
- The autofluorescent dots are an interesting feature of this Methanosarcina isolate, anaerobic cryostocks were made and could be revived for further experiments – also a metagenome of the bottle is available to look for differences in the metagenome to *Methanosarcina vacuolata*. 

Magdalena Mayr, 2016
VI.  Acknowledgements

I am very thankful for the wonderful course this summer, my thanks go to our course directors Jared Leadbetter and Dianne Newman, who were always excited about giving advice, sharing knowledge and spreading enthusiasm! I would also like to thank the whole course faculty, who supported and encouraged us during the whole course with their knowledge and never ending energy. All TAs were just generally awesome, supporting us during every second of the course. I especially thank Dima for designing the Parcu-FISH probe and FISH advice, and Kate for being as excited as me for the small bacteria and for making all the molecular work possible. Of course I thank Group Awesome for being extremely awesome and all the students of the course, who made the time during the course, and during the few breaks we had so wonderful!

I would also like to thank my advisors Helmut Bürgmann and Martin Ackermann who made me aware of the course.

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VII.  Literature


**VIII. Supplementary material**

> Marinilabiliaceae_conesus_sequence

GGGTTTAAGGCTGAGGCGAACATTGGAATGGTAGTAGTGGTGAAATATGCAGCTTAACCTTGAATT
GCCATTGATACGATTTGAGTAGGAGTGGCAGAATGTTAGTGCTAGCGGTGAAAAGTC
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**100X FW Base**

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<td>1 M Sodium sulfate</td>
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<td>0.6 M</td>
<td>1 M NH₄Cl solution 15 mL</td>
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**See water complete medium** – Dilute “SWC” Sea Water Complete
1X SW (seawater base) 1000 mL
bacto tryptone 1 g
yeast extract 0.5 g
glycerol 1 mL
1 M MOPS, pH 7.2 5 mM

**1x Seawater base (SW) (per 20L)**
Component, Amount, formula weight, Stock Conc., Media Final Concs.

**1M MOPS buffer**
Component, Amount, formula weight, Stock Conc., Media Final Concs.
MOPS free acid, 209.26 g, 209.26, 1000 mM, 5-20 mM
5M NaOH 100 mL 500 mM

**Freshwater Acetate + Methanol Degrading Purple Nonsulfur-Phototrophs (in this case methanogens)**
-Autoclave in Widdel vessel
-Cool under stream of N₂/CO₂ (80%:20%) gas
Deionized water 2.9 liter
100X FW Base 30 mL
Sodium Acetate 15 g
1 M NH₄Cl solution 15 mL
1 M Sodium sulfate 0.6 mL
100 mM K phosphate, pH 7.2 30 mL
1 M MOPS Buffer, pH 7.2 4 mL
Trace Elements 3 mL
-Dispense into sterile Pfennig bottles leaving pea sized bubble, cap tightly.
-Inoculate with ~3-5 g of Trunk River Pond Sediment or Soil sample.
After 2-4 hours of dark adaption: incubate under the assigned light regime.

**Freshwater Sulfur-Phototrophs**
-Autoclave in Widdel vessel
-Cool under stream of N2/CO2 (80%:20%) gas
-Dispense into sterile Pfennig bottles leaving pea sized bubble, cap tightly.
-Inoculate with ~3-5 g of Trunk River Pond Sediment or Soil sample.
After 2-4 hours of dark adaption: incubate under the assigned light regime.

**Multipurpose Plate Isolation**
-After cooling, add:
Multivitamin solution 3 mL
1 M Sodium bicarbonate 10 mL
DCMU 50 mg
Methanol (fresh bottle) 2ml

**Freshwater (“FW”) Base (per liter)**

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**1000x 13-Vitamin Solution**

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- Titrate with 5 M NaOH (5-10 drops) until dissolved.
- Filter sterile (0.2 μm filter) and refrigerate in the dark in 10 mL aliquots.