Scientific Report of the Exchange Grant

Growth and study of lithotrophic Fe-oxidizing bacteria at the Bigelow Laboratory for Ocean Sciences (USA)

within the framework of the ESF activity entitled
The Functionality of Iron Minerals in Environmental Processes

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1. Purpose of the visit

The aim of my visit to Bigelow Laboratory for Ocean Sciences in the US was the acquisition of knowledge in isolation and cultivation methods of microaerophilic Fe-oxidizing bacteria. The geomicrobiology department in the above mentioned laboratory under the supervision of Dr. David Emerson is working with great success in this field since many years. Different isolation approaches and techniques that have been developed within this group are useful for studies in the geomicrobiology group of Prof. Dr. Andreas Kappler at the University of Tuebingen in Germany. In this respect, samples from projects at the geomicrobiology group at the University of Tuebingen as well as samples directly taken from the Boothbay Harbor region were analyzed and selected for the isolation of Fe-oxidizing bacteria in the Bigelow Laboratory.

Bacteria are known to play a major role in iron cycling and – depending on the environmental conditions – they can mediate Fe(II) oxidation or Fe(III) reduction, and they can outcompete the abiotic oxidation/reduction rates. Experiments in the laboratory often require the use of bacterial cultures rather than environmental samples in order to achieve reproducible experimental conditions that allow for gaining insights into mechanisms that are involved in the oxidation/reduction of iron and in the precipitation of Fe mineral phases. The isolation and cultivation of bacterial strains from the environment, however, is still one of the major challenges of environmental microbiology and geomicrobiology. The majority of microorganisms that can be found in the environment so far have not successfully been cultured in the lab. Microaerophilic Fe-oxidizing bacteria in particular have been proven to be difficult to be isolated because of the special chemical conditions they need for growth. Dr. Dave Emerson's group has developed and successfully applied different approaches to achieve chemical conditions which preferentially support the growth of microaerophilic Fe-oxidizing bacteria, such as the use of "gradient-tubes" and "gradient-plates". These techniques provide two opposing artificial gradients of O_2 and Fe(II) in which bacteria can grow under their preferred conditions.

2. Description of the work carried out during the visit

Cultivation and isolation techniques for Fe-oxidizing bacteria were applied to samples from both fresh- and seawater environments. For these experiments, environmental samples from ongoing projects of the geomicrobiology group at University of Tuebingen as well as fresh samples from the Boothbay region were used. The description of the work is divided into the cultivation of freshwater and seawater strains.

Fresh water cultivation and isolation techniques

Sample description

The following list presents briefly the samples used for the enrichments and isolations:

- Bacterial mat sampled at Segen Gottes mine (Figure 1, a) in the Black Forest (Germany)
- Bacterial mat sampled at the stream lakeside drive (Figure 1, b) in the Boothbay region (Maine, USA).
- Bacterial mat sampled at Gonzen mine (Switzerland, Figure 1, c)
- Samples from the hyporheic zone of the fresh water stream Steinlach in Tübingen (Germany, no picture shown here)

Figure 1: (a) *Sampling site of bacterial mat at Segen Gottes mine (picture from Martin Obst), (b) bacterial mat next to the stream lakeside drive in Boothbay region and (c) bacterial mat floating on the surface of a flooded shaft in the Gonzen mine in Switzerland.*

Some geochemical data characterizing the sampling sites are listed in Table 1.

Table 1: *Geochemical data from the different sampling sites.*

Sample site	**ID**	**pH**	**Fe (tot)**
Gonzen mine	SB	7.2	Not determined
Segen Gottes mine	MOSG_2	6.37	
Steinlach river	SH11_NR	7.5	24 µmol/g

The samples used from the geomicrobiology group of University of Tuebingen were collected in sterile tubes and were stored at 4°C during transport to the Bigelow Laboratory (Maine, USA).

Cultivation and isolation methods that were applied

Gel stabilized gradient tubes

All samples were used to inoculate gel-stabilized gradient tubes that were prepared according to Emerson and Floyd (2005). 3.75 ml modified Wolfe's mineral medium (MWMM) amended with trace metals and vitamins and

stabilized with low melt agarose covered a thin layer (0.75 ml) of a 1:1 mix of FeS and MWMM stabilized with high melt agarose. This created a gradient of O_2 from the top and Fe(II) supply from the bottom of the tube (Figure 2). The pH was stabilized in a range between 6.1 and 6.4 using 10 mM MeS buffer and $NaHCO_3$. As an alternative buffer system 20 mM sodium bicarbonate and CO_2 gas were used. 100 µL from the original sample were used as inoculum for the first set of tubes. Since bacterial growth in the gradient tube was confirmed after a few days, a dilution series was carried out from 10^{-1} to 10^{-7} for all samples. Those were subsequently incubated at 26°C in the dark. Cultures were diluted and transferred within 4-6 days into fresh gradient tubes. Growth was checked under the microscope after staining the cells with Syto 13 (green fluorescent nucleic acid stain). Samples were transferred 7 times with dilutions from 10^{-1} to 10^{-7} for each step.

Stalk and sheath forming Fe(II)-oxidizing bacteria often do not form these extracellular polymer structures when growing in agarose. Thus, gradient tubes were also prepared as described above but without adding agarose to the top and bottom layers. At the same time the lack of agarose eliminates heterotrophic growth. Therefore, half of the tube was filled with the top layer and was followed by a bottom layer of FeS, which was carefully added to the bottom of the tube using a Pasteur pipette. The bottom layer settled down and did not mix with the top layer. 100 µL inoculum were added throughout the length of the tube so that it was distributed along the entire Fe(II)-O_2 gradient.

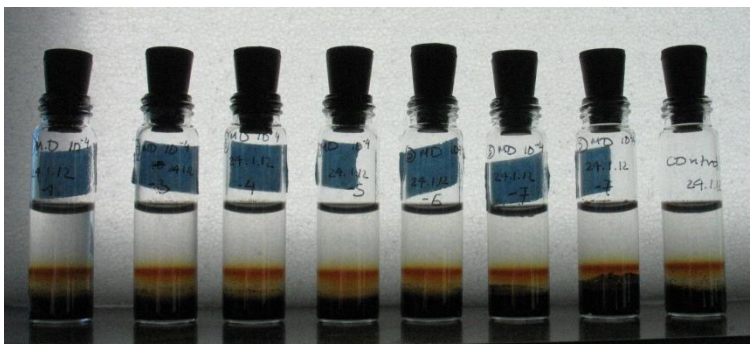

Figure 2: *Gel stabilized gradient tubes of the sample MOSG.*

Liquid cultures with $FeCl_2$

A further technique that was used for enrichment or isolation of microaerophilic Fe-oxidizing bacteria was enrichment in vials filled with liquid MWMM and no agarose. Serum vials of 15 ml volume were used and filled with 10 ml MWMM (Figure 3). The pH was adjusted as described previously for the gel stabilized gradient tubes between 6.1 and 6.4. Vials were flushed with N_2 and sealed. After autoclaving, the vials could be stored until use. Before inoculation, 100 µL sterile prepared 100 mM $FeCl_2$ were added in the vials using a 1 ml syringe. After mixing of the solution, 2-5% (v/v) O_2 was injected with a 1 ml syringe. O_2 was previously autoclaved in an empty sealed serum bottle.

Figure 3: *15 ml vials filled with MWMM and $FeCl_2$ used for isolation and cultivation purposes.*

Gradient plates

The cultivation of environmental samples in gradient plates is another useful approach for enrichment and isolation of microaerophilic Fe-oxidizing bacteria. This method is similar to the preparation of gel stabilized gradient tubes method but differs in the use of petri dishes instead of glass tubes and the absence of agarose in the top layer. The bottom layer of the plate is composed of 8 ml of a 1:1 mixture of FeS and MWMM as well as 1% high melt agarose while the top layer contains 15 ml MWMM medium amended with vitamins and trace metals. After inoculation the gradient plates were stored in a jar that provided a microaerophilic environment using BBL Campypak Plus microaerophilic system envelopes (Becton, Dickinson and Co., NJ; 5 to 15% O_2, Figure 4). This technique is particularly useful when high cell densities are required. For example, gradient plates were used for DNA extraction of the MOSG isolate used to amplify and sequence the 16S rRNA gene. This process is described below.

Figure 4: *Gradient plates in the jar providing a microaerophilic environment using BBL Campypak Plus microaerophilic system envelopes.*

Molecular biology methods

8 gradient plates were prepared and incubated for 5 days in order to obtain enough cells for DNA extraction. The liquid fractions of two plates each time were mixed in a 50 ml falcon tube and centrifuged. The pellets of all centrifuged tubes were subsequently combined. DNA was extracted using the PowerMax soil DNA isolation kit. The extracted product was distributed in four 2 ml centrifuge tubes and concentrated in a DNA speed Vac (DNA 110 Savant) for 1.5 hours. The end volume of the tubes was between 260 and 290 µL DNA product. An isopropanol precipitation according to the Quiagen isopropanol precipitation protocol (http://www.qiagen.com/literature/qiagennews/0399/993prah.pdf) followed, in order to purify the concentrated nucleic acid product.

A PCR reaction was performed in order to amplify the 16S rRNA gene of the extracted DNA. The reverse primer 1492R and the forward primer 27F (Lane, 1991) as well as the AmpliTaq Gold Master Mix (Applied biosystems) were used for the master mix. A PCR reaction was carried out for both 2.5 and 0.5 µL template added to the master mix. Subsequently a PCR clean up with the QIAquick PCR Purification kit took place. The concentrations were measured using a Nanodrop Spectrophotometer and the Quant-iT ™ dsDNA High-Sensitivity Assay Kit (Invitrogen detection technologies). The DNA product was sent to the Roy J. Carver Biotechnology Center (http://www.biotec.uiuc.edu/index.html) at the University of Illinois for 16S rRNA sequencing using the following sequencing primers: 27F, 907R, 519F, 1492R.

Sea water cultivation and isolation techniques

Sample description

PVC pipe samplers containing subsamplers (Figure 5, a and b) with 1018 mild steel coupons as described by McBeth et al. (2011) were placed on the seabed (5-7m depth, Figure 5) in West Boothbay Harbor (43.84443°, 69.64095°), in order to enrich marine Fe-oxidizing bacteria. The incubation time of the samplers was two weeks between the 10.1.2012 and the 25.1.2012. Water temperature was at sampling time 4.5°C and pH 8.

Figure 5: *PVC pipe sampler (b) and subsampler (a) used for the observation of corrosion processes by neutrophilic, microaerophilic iron-oxidizing bacteria. Image c shows the dock from which the sampler was placed on the seabed (picture from Joyce McBeth).*

Cultivation and isolation techniques

Aliquots of 100 µL of the collected sample were used to inoculate petri plates within a few hours after sampling. Plates contained autoclaved zero-valent iron (ca. 60 mg of 200-mesh with 99% Fe(0); Alfa Aesar, Ward Hill, MA) powder and autoclaved artificial sea water as described in Emerson and Floyd (2005). Plates were stored in a sealed acrylic jar with a BBL Campypak Plus microaerophilic system envelope (Becton, Dickinson and Co., NJ; 5 to 15% O2). The highest dilution which contained stalks (confirmed by microscopic observations) was transferred and diluted from 10^{-1} to 10^{-7} in new plates every 2-5 days. Samples were stored in a sealed acrylic jar with a BBL Campypak Plus microaerophilic system envelope (Becton, Dickinson and Co., NJ; 5 to 15% O_2) at room temperature. During the first three transfers puffy orange structures developed due to formation of stalks by the bacteria. Those clotted particles were observed and analyzed using phase contrast microscopy in order to verify the stalk formation.

A washing technique prior to transfer of particles, initiated by Krepski et al. (2011) was tested. Therefore a single puffy particle was selected from the sample enrichment with an autoclaved pasteur pipette. The selected particle was washed carefully five times in autoclaved artificial sea water medium and subsequently diluted 7 times (by 1/10). Diluted samples were incubated 2-5 days and checked for growth. For the last transfers, due to lower cell density no puffy structures could be observed macroscopically. Therefore a micromanipulator (Eppendorf, TransferMan® NK 2) was used to select one tiny particle. The selected partile which contained stalks was diluted and transfered in 5 new plates. The microscope was previously sterilized with 70% alcohol and all other parts were autoclaved or UV sterilized. The sample was transfered 6 times in total. After the 5th transfer, the culture was streaked out on ASW-R2A agarose plates to test the culture for the presence of heterotrophic bacteria.

3. Description of the main results obtained

Fresh water cultivation and isolation techniques

Microscopy observations

All samples were observed under the microscope prior to inoculation of the gradient tubes. Plenty of stalks and sheaths were found in the SB (Gonzen mine, Switzerland) sample. Characteristic stalks were abundant in MOSG samples (Figure 6) while sheaths could be detected in the LD samples collected in Boothbay region. None of these extracellular polymer structures could be seen in the SHR_N11 samples. Some important geochemical data for the samples are listed in table 1.

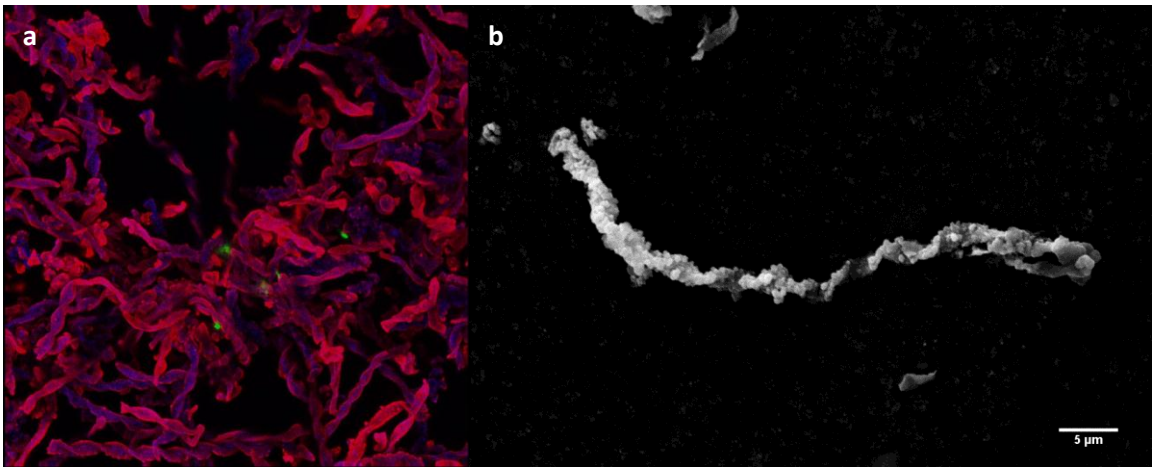

Figure 6: *Twisted stalks in MOSG samples shown with confocal laser scanning microscopy (a) and scanning electron microscopy (b). Sample was stained with Syto 9 and Lectin WGA conjugated with Alexa 555. Images were made at University of Tuebingen after the end of the visit at Bigelow Laboratory.*

Cultivation and Isolation

Two days after the first inoculation of the LD and the MOSG samples, an oriented growth outward of the inoculum and vertically to the formed band in the gradient tube, was distinctive. However, no characteristic structures such as sheaths and stalks were observed in the inoculated gradient tubes. In the two other samples (SG and SH11_NR), no specific growth was observed macroscopically. Nevertheless observations with fluorescence microscopy showed the presence of microbial cells in all gradient tubes that were inoculated. Therefore all the samples were transferred in new series of gradient tubes. After the second transfer no distinguishable growth form or characteristic band formation could be observed anymore, while growth could be proven with fluorescence microscopy by staining the cells with Syto 13. After 5 transfers, R2A plates were prepared and streaked out for all samples. Heterotrophic growth was detected in the samples SG, LD and SH11_NR after 3-4 days, and thus these isolation attempts were abandoned. However, no colonies developed on the R2A plate of the MOSG sample over 10 days, while cells were obviously growing in the gradient tube. Moreover cells in the gradient tube of MOSG had a uniform morphological appearance, suggesting the purity of the culture. Therefore this sample was transferred in gradient plates for DNA extraction and sequencing purposes.

Summing up, the cultivation and enrichment of bacteria from environmental samples using the previously mentioned gel-stabilized gradient tube approach was successful in all cases. However, only in one case an isolation of a bacterial strain was successful. This could be the result of the sample composition as well as the age of the samples. Experience has shown that the fresher the sample is the higher is the possibility to isolate an Fe-oxidizing bacterium.

DNA of the MOSG culture was extracted and amplified in order to obtain the 16S rRNA gene sequence. The culture appeared to be a pure strain, and was found to be a 99% match for *Polaromonas* sp. R-36500 partial 16S rRNA

gene. This strain belongs to the group of *Betaproteobacteria*. The 16S rRNA sequence is available in a supplementary part of this report. It is not known if MOSG gains energy from the iron oxidation directly. What is interesting is that GM1, which is a Polaromonas related species, can oxidize arsenite (Osborne et al., 2010) while EDX data of the sampling site in Segen Gottes obtained at the University of Tuebingen, showed arsenic content in the bacterial mat as well (data not shown). However there is no evidence of iron oxidation by Polaromonas species and further research is needed in this field. The strain was therefore transferred to Tuebingen where it is growing on FeS and used for further studies.

Additional experiments performed in the laboratory

- In addition to the experiments described above, laboratory cultures of previously isolated strains such as Br-1 (Weiss et al., 2007) and ES-1 (Emerson and Moyer, 1997) were grown to learn and test the experimental approaches. The strains were cultured in gradient plates, gradient tubes and in $FeCl_2$ liquid vials. The cultivation was successful in all cases while no heterotrophic growth could be detected on R2A plates.

- Samples of various projects were used for isolation purposes as well. The isolations was however not successful, most probably due to the age of the samples.

- In the gradient tubes without agarose bacterial growth was only observed for the isolated strain ES-1 (Emerson and Moyer, 1997), which was found to grow at the FeS – medium interface.

- The LD and MOSG samples were additionally transferred in vials with $FeCl_2$ as Fe-source, where no agarose was present in the medium, in order to eliminate heterotrophic growth. However, no growth was observed for the MOSG samples in those vials while cell growth was evident for the LD sample. Interestingly the cell morphology of the LD sample growing heterotrophically on R2A plates and the one growing in the vials with $FeCl_2$ as iron source, was very similar, leading to the assumption that it could be the same strain. Therefore heterotrophic cells of one colony growing on R2A plates were used to inoculate vials with liquid MWMM and 100µM $FeCl_2$. The same was done in vials without $FeCl_2$ in order to test the influence of growth conditions on the culture. The culture was growing in both conditions and is still growing in vials with $FeCl_2$ in case further studies are needed.

Sea water cultivation and isolation techniques

Sample observations

The reddish color of precipitates in the water, surrounding the mild steel coupons (Figure 5) that were used for the experiment, revealed the iron oxidation after two weeks of incubation. Fluorescence microscopy observations showed various bacterial morphologies in the original sample while characteristic twisted stalk formations could be seen with bright field microscopy (not shown in this report). It is known from previous studies (McBeth et al., 2011) that dock D2b-C6 clone [Gen-Bank accession no. HQ206656, Figure 7) is present in this sample, which is known to produce helical stalks.

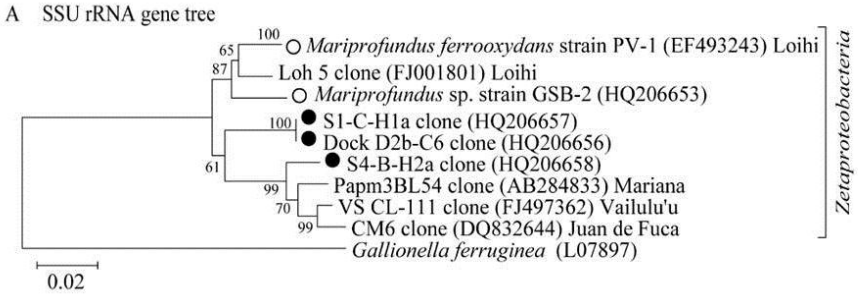

Figure 7: *Phylogenetic tree showing the Dock D2b-C6 clone (McBeth et al., 2011)*

Cultivation and isolation techniques

One day after the first inoculation of the sample in the plates, red puffy structures (Figure 8, a) started developing. Microscopic observations of those particles showed a high abundance of helical stalks, which were probably responsible for the formation of the macroscopic structures. Growth of the stalk forming bacteria was demonstrated as cells attached on stalks could be seen dividing (Figure 8, b). After the 6th transfer, samples were streaked on artificial seawater R2A plates. As no heterotrophic growth was detected, DNA was extracted from the culture and amplified for 16S rRNA analyses. The results showed that it is a pure culture and its 16S rRNA gene sequence is identical to the *Zetaproteobacterium* clone Dock D2b-C6. The bacterial strain was successfully isolated from the environmental sample. The strain was called DIS-1 and is most probably a chemolithoautotrophic, microaerophilic Fe-oxidizing bacterium as it grows without an organic carbon source on zero-valent iron. It is closely related to the Fe-oxidizing bacterium *Mariprofundus ferrooxydans* within the *Zetaproteobacteria* (Figure 7) which produces similar extracellular helical structures as DIS-1. However DIS-1 is the most novel isolate that has been obtained since isolation of *Mariprofundus ferrooxydans.* Another culture, that seemed to be pure in the ASW medium, is still being analyzed.

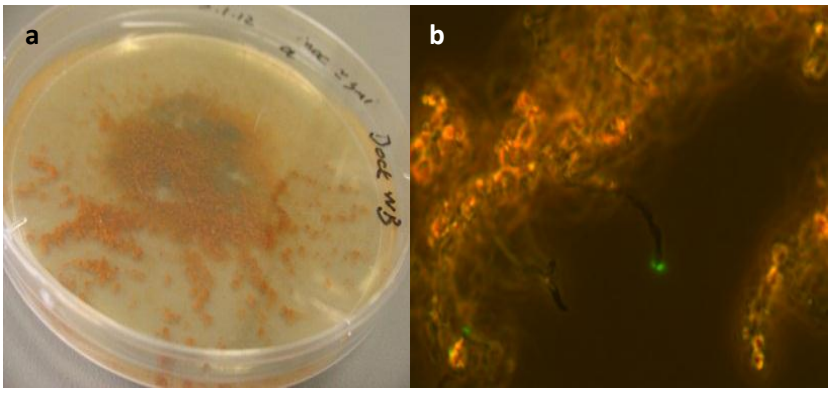

Figure 8: *Characteristic puffy particles formed due to stalks in the ASW medium (a), microscopical observation of the puffy particles. A dividing cell on a stalk can be distinguished (b).*

4. Future collaboration with host institution (if applicable)

Future collaborations of the geomicrobiology group of both institutes (Bigelow Laboratory for Ocean Sciences and AG Kappler at the University of Tuebingen) are always possible, as projects taking place in both institutes are closely related and common interests can lead to future cooperative work.

5. Projected publications / articles resulting or to result from the grant (ESF must be acknowledged in publications resulting from the grantee's work in relation with the grant)

A future publication is possible regarding the new isolated bacteria, especially the *Zetaproteobacterium* DIS-1.

6. Other comments (if any)

My visit in Bigelow Laboratory for Ocean Sciences helped me extend my knowledge in lab techniques. I learned cultivation and isolation methods of neutrophilic, microaerophilic Fe-oxidizing bacteria, bright field and epifluorescence microscopy and the use of a micromanipulator system. Methods that I learned there can now be taught and applied in several projects at the geomicrobiology group in Tuebingen. Additionally some of the experiments from which no isolate could be obtained, will be repeated with a very fresh sample at my home University. Additionally I had the opportunity to receive an impression of diverse scientific projects taking place at the institute and meet brilliant scientists from whom I could be inspired and could obtain useful advices and knowledge for the future.

Acknowledgements

I want to thank the whole geomicrobiology group at the Bigelow Laboratory for Ocean Sciences: Dr. David Emerson, Dr. Joyce McBeth, Dr. Emily Fleming, Dr. Erin Field and Dr. Michael Preston for their supervision, help, advice and the fantastic time in the lab.
Additionally I want to thank my supervisors at University of Tuebingen Prof. Dr. Andreas Kappler, Dr. Sebastian Behrens and Dr. Martin Obst for their great help and support during the entire time before, during and after my visit.
Finally I want to thank Ankita, Martin, Steffen, Caroline, Nina and Nicole who gave me samples of their projects in order to test and learn the methods.

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wetland plants and description of *Ferritrophicum radicicola* gen. nov. sp. nov., and *Sideroxydans paludicola* sp. nov. Geomicrobiology Journal. 24: 559-570

Appendix

16S rRNA sequence of Polaromonas species isolated from MOSG

CGTGGTATCGCCCTCCTTGCGGTTAGGCTAACTACTTCTGGCAGAACCCG
CTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACC
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16S rRNA sequence of dock D2b-C6 isolate

CCTCTCCGAAGAGTTAGCCNGGCGGCTTCTGGTGCAACCGATTCCCATGG
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