Different biofilm-forming purple sulfur bacteria enriched from Trunk River

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Abstract

Three different types of biofilm were developed on the bottles of purple sulfur bacteria enrichments. The original inoculum is a piece of sea grass covered with purple biofilm that collected from Trunk River during the course. Microscopy imaging showed that two of the three biofilms were apparently composed of two major species. MonoFISH probing supports the recognition of purple sulfur bacteria as Chromatium in the class of gammaproteobacteria which grow together with a deltaproteobacteria species. Such a combination of Chromatium colonize with deltaproteobacteria species is also originally present in the purple biofilm on sea grass. Further work is needed to investigate the potential interactions between these two species.

Introduction

Purple sulfur bacteria are photosynthetic anearobes in the phylum of Proteobacteria (Fowler et al., 1984), which is capable of fixing carbon dioxide with sulfide other than water as the electron donors. Since oxygen is not produced during their photosynthesis these purple sulfur bacteria are also known as anoxygenic photoautotrophs. Most purple sulfur bacteria synthesize bacteriochlorophyll and carotenoids as their light-harvesting pigment complex (Iba et al., 1988). Because their photosynthesis requires anoxic condition and sulfide, these purple sulfur bacteria are often found in organic rich aquatic environments where sulfate reducing heterotrophic bacteria thrive.
Both planktonic and benthic species of purple sulfur bacteria exist in different sulfidic environments. In the habitat of stratified meromictic lakes with external sulfate input, such as Green Lake, Mahoney Lake and Lake Cadagno, the phototrophic chemocline microbial communities are often dominated by planktonic purple sulfur bacteria living on sulfide diffused up from organic rich sediment (e.g. Meyer et al., 2011; Hamilton et al., 2014). However, in microbial mats developed in coastal marshes, like Sippewissett and Trunk River, the cell of purple sulfur bacterial can be in close contact with sulfate reducing bacteria and other communities. The thin purple biofilm formed on decaying sea grass in Trunk River provides a very simple and easy access community to study the interaction between purple sulfur bacteria and other microbes.

**Materials and Methods**

**Sample collection**
A piece of sea grass covered with purple biofilm was collected from the pond area of Trunk River. Sample was kept in a 50 ml falcon tube with in situ water under room temperature until inoculation.

**Enrichment incubation**
A modular freshwater media was used for the sulfur phototrophs enrichment. To prepare the media, 22 g sodium succinate, 20 ml 1M NH₄Cl solution, 1 ml 1M sodium sulfate, 40 ml 100mM potassium phosphate (pH 7.2), 5 ml 1 M MES buffer (pH 5.5), 4 ml trace elements and 40 ml of 100X fresh water (FW) base that consist of 100 g NaCl, 40 g MgCl₂·6H₂O, 10 g CaCl₂·H₂O, 50 g KCl per liter were added in 3.9 liter of deionized water, and then autoclaved in widdel vessel. After autoclave, the media was cooled under stream of N₂/CO₂ (4/1) gas. After cooling, add 4 ml of multivitamin solution, 280 ml 1 M sodium bicarbonate, 40 ml 1 M thiosulfate, 4 ml 1 M sulfide and 50 mg DCMU for inhibiting oxygenic photosynthesis. Collected sample
was then transferred into a pfennig bottles with the prepared media, caped tight and placed in dark for 2 hours and then incubated under 850 nm LED in a dark chamber.

**Shake tube series**

3 ml of 3% agar was autoclaved in each shake tubes and kept in 50 °C water bath, and added another 9 ml of preheated liquid media as described above. 100 μl of original enrichment (after 15days of incubation) was transferred into a shake tube and mixed well. And then, 1ml of the first shake tube culture was quickly transferred into a dilution series until 10⁻⁷. Inoculated tubes were quickly cooled with ice bath and incubated upside down under 850nm LED.

**Enrichment spectroscopy**

Enrichments with biofilm development on bottle were analyzed using the Spectral Evolution spectroradiometer (model SR-1900) and DARWin software (Spv1.2.5093). 4 to 6 replicate absorbance spectra were analyzed for each bottle with a bottle of original media as blank reference.

**monoFISH**

A few pieces of biofilm were transferred from the pfennig bottle with a glass pipette into 2 ml eppendorf, and washed with sterile PBS for three times. Washed cell was fixed with 4% paraformaldehyde (PFA) at room temperature for 2 hours. Hybridizaiton of fixed biofilm with selected probes, DELTA495a, DELTA495b, GAM42a, EUB338-1 and NON338, was carried out on glass slide according to the standard monoFISH protocol (Manz et al., 1992). After hybridization samples were mounted on a new glass slide for microscopy.

**Results**

1. The composition of original purple biofilm on sea grass.
A small piece of freshly collected sea grass was mounted on glass slide and checked under microscope. As shown in Fig. 1, two major types of microbial cells cover the surface of decaying sea grass. One is the purple sulfur bacteria, likely Chromatium species (~3μm), with multiple intercellular sulfur granules, and the other is smaller rod shape of cells colonize tightly together with the purple sulfur bacteria. Notably, most purple sulfur bacteria occurred in the form of clumped aggregates. Different types of green algae and cyanobacteria species were observed showing red and blue autofluorescence under UV excitation. Filamentous Beggiatoa species, dinoflagellates and diatoms were also found in the biofilm.

**Figure 1.** Piece of sea grass and microscopic image showing purple sulfur bacteria and other microbes colonized sea grass surface (left panel), and the bottle of original inoculum after 15 days, and microscopic image showing purple sulfur bacteria and other microbes forming complex aggregates (right panel).

**2. The composition of purple biofilm after 15 days of incubation.**

After 15 days of incubation the media was still clear, but big clumps of microbial cells accumulated at the bottom of bottle (Fig. 1). Purple sulfur bacteria showing different morphology were enriched, and most of them filled with clear sulfur granules inside the cell. Other kinds of rod shape cells were also observed attaching to the sulfur bacteria communities.
3. The composition of purple biofilms developed from single colonies

After one week of incubation, disc shape of single colonies grew in the shake tube with more than $10^{-5}$ times of dilution. Three types of single colonies, dark purple, light purple and white, were observed. After removing the top 1 cm of agar in the $10^{-6}$ times of dilution tube, each color of single colonies were transferred into fresh liquid media with a glass pipette under nitrogen gas flow. After another week of incubation, different biofilms developed on the wall of each bottle (Fig 2). Both enrichments from the dark and light purple colonies grew homogenous thin film, but the bottle of white colony has thicker biofilm accumulated on the light side. The spectra analysis of the three biofilm enrichments and another planktonic enrichment as reference show similar but slightly different absorption. Both bacteriochlorophyll-a (absorption at 397, 799 and 850 nm) and carotenoids (absorption between 450 ~ 550 nm) were detected (Fig. 2).

![Figure 2. Spectra absorption of three bottles of enrichments inoculated from single colonies develop purple biofilms after 7days of incubation. And, another planktonic enrichment from the trunk river sand inoculum.](image)
Under microscope, different cell morphology of purple sulfur bacteria indicate probably different species enriched in each bottle. The enrichment from white colony picked from shak tube has three major species in the liquid of earlier stage incubation, but developed later the purple sulfur bacteria dominated biofilm on the glass.

![Microscopy showing different cell morphology of Chromatium in four enrichments.](image)

Bright field microscopy of the dark and light purple biofilms revealed hybrided communities with two major species, the sulfur granule containing Chromatium and another smaller rod shape of bacteria (Fig. 4). Carbonate crystal grew on the white colony biofilm suggested increasing pH due to the consumption of $H^+$ by autotrophic metabolism of Chromatium.
Figure 4. Microscopy images showing the hybridized biofilm of dark and light purple enrichments. Carbonate crystal grew on the white purple biofilm. After acid treatment, gas bubbles was generated from dissolved carbonate.

MonoFISH experiment supports the tentative recognition of purple sulfur bacteria as Chromatium species in the class of Gammaproteobacteria. The other co-living rod shape microbe is a Deltaproteobacteria species.

Figure 5. Microscopy images showing DAPI and proteobacteria probes, GAM42a and DELTA 495a+b, labeled cells with monoFISH experiment.
Discussion

Three biofilm forming purple sulfur bacteria are enriched from the same inoculum of trunk river sea grass. Different cell morphology suggested distinctive species of Chromatium. Interestingly, two biofilm are clearly mix communities of Chromatium and another Deltaproteobacteria. Chromatium species are photoautotrophs that oxidizing sulfide. The co-living Deltaproteobacteria that forming a tight hybrid biofilm must have a close interaction with the Chromatium. One scenario is that the Deltaproteobacteria is heterotrophic sulfate reducer. In this case, the Deltaproteobacteria can feed on the oxidized sulfur (sulfate) and organic matter produced by Chromatium, and in return supply the Chromatium with reduce sulfur. A potentially coupled carbon and sulfur cycle can take place on the purple biofilm. Alternatively, another scenario is that the Deltaproteobacteria can disproportionate the high concentration of thiosulfate in the media and generate both sulfide and sulfate. In this case Chromatium can also take advantage of the sulfide supply from co-living Deltaproteobacteria. Similar cases can also exist in Trunk River on the purple biofilm covered sea grass. Further studies and cultivation experiments with different media composition need to be conducted for better understanding of the interaction between the purple sulfur bacteria and co-living Deltaproteobacteria.

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References:


