Study of phylogenetic consistency, structure and origin of the pink berries in Great and Little Sippewissett salt marsh

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Abstract

In the past 20 years pink microbial aggregates from the Little and Great Sippewissett salt marsh have been studied in the microbial diversity course, Woods Hole, Massachusetts. Clone libaries revealed that the aggregates may be dominated by two main phylotypes 1) *Halochromatium* sp., belonging to the purple sulfur bacteria of the Gammaproteobacteria, and 2) *Desulfofustis* sp., characterized as a sulfate-reducing bacterium in the Deltaproteobacteria. Furthermore, the biomass of the aggregates was found to be dominated by two morphotypes: 1) large, spherical and pigmented cells and 2) smaller, rod-shaped bacteria. The physical arrangement and their phylogenetic relationship to the Gamma- and Delatproteobacteria, respectively, have been demonstrated.

In this study, cluster-specific FISH-probes for the *Halochromatium*-related and the *Desulfofustis*-related phylotypes of the berries were designed to serve the confirmation that these phylotypes are forming the main biomass of the berry aggregate. Furthermore, they were used for the specific description of cell arrangement within the aggregate and were tested on water and sand samples in order to reveal the putative presence of free-living stages of the phylotypes. By this, putative early stages of aggregate formation were found in pink sand banks, giving insights into the potential origin of the berries. Isolation of the putative sulfate-reducing bacteria from the berries should provide a tool for studying the metabolites used and products excreted by these organisms, which could help to understand the physiological interconnection of the two dominating organisms in the aggregate.

Introduction

Large, macroscopic microbial aggregates, so-called 'pink berries' from Little and Great Sippewissett salt marsh, Massachusetts, have been studied in the microbial diversity course at MBL, Woods Hole, since more than twenty years. Clone libraries of the 16S rRNA genes have been generated (Banin, 1997; Scott, 2007; Wilbanks *et al*., 2010) in order to identify the phylotypes present in the aggregates. Two main phylotypes have been identified: 1) *Halochromatium* sp., belonging to the purple sulfur bacteria of the Gammaproteobacteria, and 2) *Desulfofustis* sp., characterized as a sulfate-reducing bacterium in the Deltaproteobacteria. The third most abundant phylotype was *Cytophaga* sp., which are heterotrophic, aerobic bacteria of the Bacteroidetes class. In a previous study (Wilbanks *et al.*, 2010), specific fluorescence *in situ* hybidization-probes for Deltaproteobacteria and *Cytophaga* spp. demonstrated the dominance of these phylotypes in the berries. The main biomass of the berries, however, is composed of bacteria that feature a large (3 µm), spherical morphology, that contain red pigments and that are considered to represent the *Halochromatium*-related phylotype. Until today, however, it has never been demonstrated that the sequences forming the two main phylogenetic clusters in clone libraries (*Halochromatium*-related and *Desulfofustis*-related) originate from organisms forming the main biomass in the macroscopic aggregates.

Assuming that these two phylotypes are also the physiological keyplayers of the berry consortium, their physical association might play a functional role. Wilbanks *et al*. (2010) suggested an internal sulfur cycle between the *Halochromatium*-related and *Desulfofustis*-related phylotypes. NanoSIMS measurements revealed the incorporation of ^{34}S into the large, round morphotypes, which were thus identified as the purple sulfur bacteria based on morphology, after incubation of aggregates with $^{34}SO_4^{2-}$. It was speculated that the other phylotype, which is assumed to be represented by the smaller, rod-shaped morphotype, performs sulfate reduction, because of their phylogenetic relation to sulfate-reducing *Desulfofustis*/*Desulfocapsa* species. Sulfate-reduction results in the formation of sulfide, which may fuel anoxygenic photosynthesis of the purple sulfur bacteria in the Sippewissett aggregates. A physical association of these two partners (large purple sulfur bacteria and small putative sulfate reducer) realizing this internal sulfur cycling

was assumed and indicated by Wilbanks *et al*. (2010), who localized the putative sulfate-reducer with a Deltaproteobacteria-specific FISH probe and found them predominantly attached to large cell clusters of purple sulfur bacteria. They described the arrangement of the large, spherical purple sulfur bacteria (detected by their autofluorescence) and the smaller, rod-shaped Deltaproteobacteria as patchy islands, surrounded by an extrapolymeric substance, containing many *Cytophaga* filaments (Wilbanks *et al*. 2010).

Large berry-aggregates found exclusively in saltwater pools in the Sippewissett marsh, however, the origin and formation of the aggregates was suggested to be in the sand banks surrounding the water pools and spartina areas. In the sand banks, single sulfur bacteria were detected that resemble the morphology of the main morphotype found in the pink aggregates and that were shown to spontaneously aggregate when shaken for a few seconds in seawater (Seitz *et al*. 1993).

Material and methods

Sampling

Aggregates were sampled from two pools in Little Sippewissett and one pool in Great Sippewissett. Pool water was collected from the first large pool in Little Sippewissett and pink sand was recovered from top layer of the tide-ways also in Little Sippewissett.

Community analysis by 16S rRNA gene pyrosequencing

Berries from two pools in Little Sippewissett and one pool in Great Sippewissett were washed three times sterile-filtered marsh water. As berries were bigger in Little Sippewissett, 6 (dataset V7) and 8 (dataset V8) berries were picked from two pools from this site and 15 of the smaller berries were picked from Great Sippewissett (dataset V6). The aggregates were crushed with a pestle (blue pipette tip closed at the front with the Bunsen burner), and were three times frozen at -20°C and thawed at room temperature. The homogenized berries were pipetted into the bead tube of the MoBio power soil DNA extraction kit (CA, USA), 60 µL of buffer C1 was added and the tube was bead-beaten for 30 seconds. The samples were then incubated

at 63°C for 5 minutes before the DNA was extracted according to manufactures instructions.

Probe design

The probe design tool of the ARB software package (Ludwig *et al*., 2004) was used to design specific probes for the two main phylogenetic clusters found in previous clone libraries from the aggregates (Scott, 2007; Wilbanks *et al*., 2010).

For the purple sulfur bacteria-phylotype, 71 full-length 16S rRNA gene sequences were selected and the probe sequence suggested by the program matched 69 sequences of these. Specificity for the target sequence cluster was tested with the SILVA database (release 102, Pruesse *et al*. 2007), the RDP and ncbi database, and no hits for sequences outside of the target cluster could be detected. The probe name is PSB-PiBe461 and its sequence is 5´-acgcccaagggtattaac-3´. A mismatch-clone to this sequence was available (*Thiodictyon bacillosum* or *T. elegans*), but was not tested in this study (single mismatch three nucleotides in from the 3´end).

For the *Desulfofustis*-related phylotype, 106 full-length 16S rRNA gene sequences were selected and the suggested probe sequence matched 52 sequences of these. Specificity for this sequence cluster was likewise tested with the SILVA database (release 102, Pruesse *et al*. 2007), the RDP and ncbi database, and also no hits for sequences outside of the target cluster could be detected. The probe name is SRB-PiBe213 and its sequence is 5´-tcctcctcgcacaaccgc-3´. A mismatch-clone to this sequence was not available.

Newly designed probes were ordered as horseradish-peroxidase-conjugate from biomers (Ulm, Germany) and diluted to 50 ng/ µL with DNA-grade water.

Embedding and cryo-sectioning of aggregates

Berries from Little and Great Sippewissett were washed three times in filter-sterilized marsh water and placed into PBS for 1 minute. The PBS was removed with a pasteur pipette and a solution of 4% formaldehyde and 0.5% glutaraldehyde (in MilliQ) was added. The aggregates were fixed for 1 hour at room temperature and the liquid was removed again with a pasteur pipette. Fresh PBS was added and removed three times to wash the berries before a 7.5% sucrose solution (in PBS) was added. The cells were incubated for 1 hour while slowly rotating at room temperature. Sucrose-solution was removed with a pasteur pipette, PBS was added and removed

again. O.C.T. Tissue Tek (Sakura, CA, USA) was added, well covering the aggregates. They were rotated for 16 hours at room temperature and then incubated standing at 4°C for 24 hours. Single aggregates were moved into cryo-freezing molds, which were then placed horizontally into liquid nitrogen for several minutes. The molds were kept at -80°C for 12 hours before placed at -20°C for two days.

Embedded aggregates were sectioned with a cryo-microtom HM 505 N into 20 µm sections and placed onto polysine-covered glass slides (Thermo Fisher Scientific Inc., Schwerte, Germany). Slides were stored at -20°C until used.

Fixation and filtration of pool water and pink sand

Pool water (36 mL) from Little and Great Sippewissett was mixed with 4 mL of 20% formaldehyde (4% final concentration) and incubated for 40 minutes at room temperature and for 3 hours at 4°C. A support-filter (0.45 µm pore size) was placed onto a filtration device before the 0.2 µm membrane filter was placed on top. Fixed pool water was filtered and filter tower was washed by filtering 20 mL fresh PBS. Filter was placed on whatman paper to dry and stored at -20°C.

Pink sand from sand banks (0.5 mL) from Little and Great Sippewissett was mixed with 0.5 mL of 4% formaldehyde (in PBS, 2% final concentration) and incubated for 40 minutes at room temperature. The sample was centrifuged at 10,000 xg for 10 minutes and the supernatant was discarded. The sand was resuspended with PBS by inverting the tube and centrifuged again. The sand was washed three times accordingly before being resuspended in 1 mL 50% ethanol (in PBS). The sand was diluted 1:10 with PBS in a volume of 750 µL, and when the sand has settled the supernatant (600 µL) was filtered as described above.

CARD-FISH

Slides with berry-sections were thawed at room temperature before sprayed with 0.4% low melting agarose (NuSieve, Lonza Rockland Inc., ME, USA) and dried at 37°C. Each slice was spotted with 25 µL of 10 mg/mL lysozyme (in 0.05 M EDTA, 0.1 M HCl) and incubated at 37°C for 1 hour. The slide was placed into a petri dish, washed twice with MilliQ and dried at 37°C. Internal peroxidases were inactivated either with 0.15% H_2O_2 in methanol for 30 minutes or with 0.01 M HCl for 20 minutes at room temperature. The slide was washed twice with MilliQ before placed in 96% ethanol for 5 seconds and let air-dry. Slides could be stored at -20°C at this

point. Slides were again sprayed with 0.4% low melting agarose and dried at 37°C. 1 µL of probe solution (50 ng/µL) was added to 300 µL of hybridization buffer and 25 µL were spotted onto each slice on the slide. Hybridization was performed in a hybridization chamber (contains tissue soaked with solution of appropriate formamide concentration) for 3 hours at 46°C. Slides were washed in prewarmed washing buffer at 48°C before being washed twice in PBS. Slides were dried for a few minutes without slices becoming entirely dry. The rims of the slides were dried with a kimwipe to prevent the amplification from running down the sides of the slide. 990 µL of the amplification was supplemented by 10 µL of 0.15% H_2O_2 in PBS and by 2 µL of Alexa488-tyramide conjugate (1 mg dye/mL). 25 µL of amplification buffer-mix was spotted per slice and amplification was performed horizontally in 50 mL reaction tubes at 46°C for 40 minutes. Slides were washed twice in MilliQ, dipped for 5 seconds into 96% ethanol and air-dried before mounted with 4:1 Citifluor:Vectashield containing 1 µg/mL DAPI. Slides were stored at -20°C before analyzed with the epifluorescence microscope or the confocal laser scanning microscope (Zeiss CLSM 700).

Hybridization with filter sections were performed according to the course protocol or as described on the SILVA homepage (www.arb-silva.de).

Cultivation of sulfate reducers from berries

The basic medium for the dilution-series agar-shakes tubes contained 1 mM NH_4Cl, 30 mM $NaSO_4$, 1 mM MOPS-buffer and 1 g Bacto agar. This basic medium was heated to 45°C and bubbled with N_2 for 30 minutes before filled 10 mL each into bulge tubes in the anaerobic chamber (while constantly stirring to get the same amount of agar into each tube). The tubes were closed with a rubber stopper and crimped before removed from the anaerobic chamber and autoclaved. Tubes were kept at 45°C after autoclaving to prevent the agar from solidifying. The medium in each tube was then completed with anaerobic, sterile supplements: 0.1 mL 100 mM K-phosphate; 0.1 mL 6 mM $FeSO_4$; 0.1 mL 0.1 M Na-bicarbonate; 1 drop vitamin stock; 1 drop trace elements, 1 drop 1 M cysteine. Seven tubes in a row were supplemented either with 0.1 mL 1 M acetate, lactate or glycolate. In one row, no electron donor was added as this row received overpressure of H_2/CO_2 after inoculation.

Liquid media was prepared in a similar way as the shake tubes. Differences included the usage of hungate tubes that were filled with 50 mL basic medium and completed accordingly with 0.5 mL/2drops of the supplement solutions.

The bottom of the tissue homogenizer (2 cm diameter) was filled about 3 cm with three times sterile-washed berries. As much water as possible was removed. The aggregates were homogenized for about 10 minutes with the pestle and care was taken to not bring too much air into the homogenate. 1 mL of the homogenate was injected into the first tube of each treatment-row, the tube was inverted tow times and the syringe was filled again with 1 mL. This first inoculated tube was placed on ice immediately and the removed 1 mL were injected into the second tube of the respective row. The tube was again inverted, 1 mL was removed, the tube was placed on ice and the 1 mL was injected into the third tube. In this manner, six tubes of the row were inoculated. The seventh was left sterile as control. Liquid media were inoculated with 0.5 mL berry-homogenate. H_2/CO_2 incubations received overpressure of 8 psi after inoculation. Tubes and bottles were incubated at 30°C with constant light.

Single colonies from shake-tubes were recovered by breaking the bottom of the tube in the anaerobic chamber and colonies were removed with a scalpel. Colonies were either streaked on agar plates (prepared as shake tubes in a 1 L bottle and poured in anaerobic chamber) or suspended in 100 µL liquid medium and inoculated into 30 mL liquid medium.

Results and Discussion

Community analysis by 16S rRNA gene pyrosequencing

The 454 dataset from pink berries collected from Great Sippewissett (V6) contains 10627 sequences and 726 OTUs with a cut-off of 97% identity. The dataset from berries sampled in the first large pool in Little Sippewissett (V7) contains 12431 sequences and 994 OTUS, and that from a smaller pool further into the back of Little Sippewissett salt marsh (V8) contains 7288 sequences and 677 OTUs, all at the same cut-off value.

The diversity of organisms within the berries of each of these sampling sites is highly similar as indicated by the comparable ratios of OTUs per amount of sequences within a sample. This can also be seen in the similar slopes of the rarefaction plots of each sample (Figure 1). Furthermore, this plot indicates that diversity is high in all samples and that sampling saturation has not yet been reached.

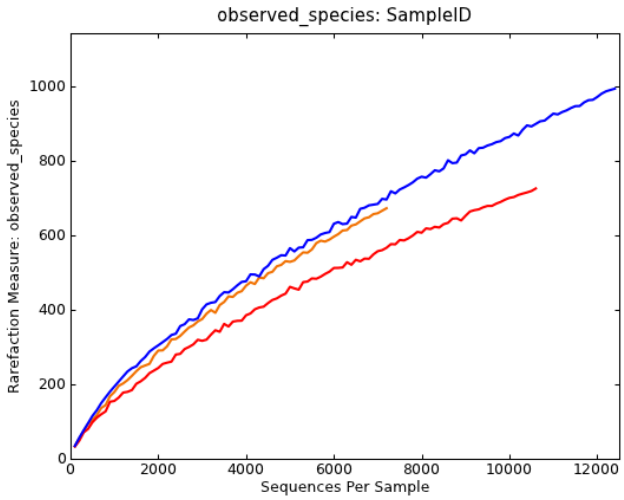
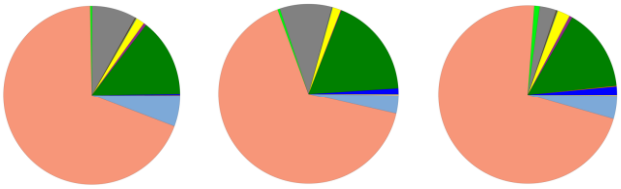
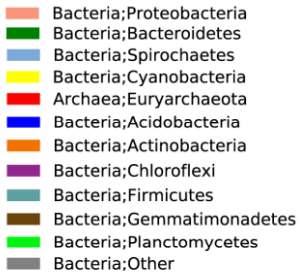
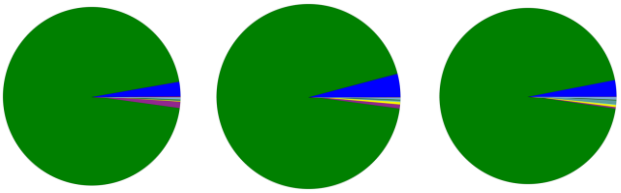
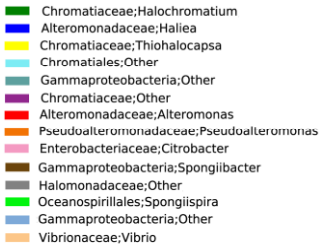
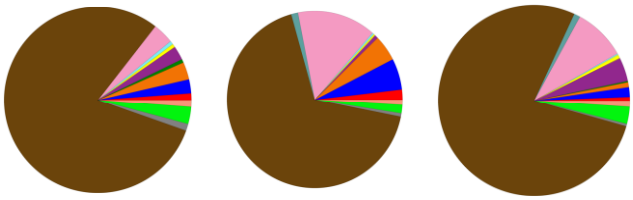
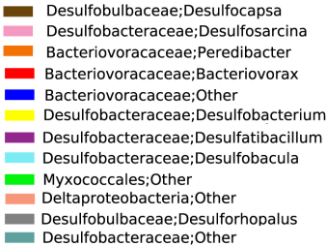


Figure 1.
Rarefaction plots of the three samples V6 (yellow), V7 (red) and V8 (blue) at 97% cut-off.

Focusing on taxonomic diversity in the three samples, it becomes evident that also the distribution of phyla among them is extremely similar. More than half of all OTUs recovered in all were related to the Proteobacteria. When broken down into OTUs found only within the Gamma- or Deltaproteobacteria, the three samples were again extremely similar in their composition. In all three samples, these two classes are dominated by OTUs showing highest similarity to *Halochromatium* and *Desulfocapsa*, respectively (Table 1). In fact, in all three samples about half of all sequences recovered were related to the genus *Halochromatium* (V6: 5006 of 10627 sequences, V7: 6676 of 12431 sequences, V8: 3731 of 7288 sequences), which was thus clearly the dominating phylotype in the pink berries independent of sampling site. Besides this, the Deltaproteobacteria were likewise dominated by a very distinct cluster of OTUs, which were closest related to sulfate-reducers of the *Desulfocapsa*/*Desulfosarcina* cluster. The latter does also contain *Desulfofustis* as one closely related genus. The short read length of about 450 nucleotides in the 454 data could cause a slightly less accurate classification of sequences on the genus level.

Table 1. Diversity of OTUs at different levels in taxonomic ranking. All three samples show a highly similar pattern. They are dominated by OTUs from the Proteobacteria and Bacteroidetes. Within the Gammaproteobacteria, all datasets are dominated by sequences closely related to the genus *Halochromatium* and within the Deltaproteobacteria sequences related to *Desulfocapsa* dominate.

sorted by	V6	V7	V8	legend
phyla				
Gamma proteo bacteria				
Delta proteo bacteria				

It can be concluded that the composition of dominant phylotypes within the berries, both from nearby sampling sites within Little Sippewissett as well as in comparison with a more distant sampling site in great Sippewissett, is highly conserved. The two dominant phylotypes used to be and seem to still are 1) purple sulfur bacteria related to the genus *Halochromatium*, and 2) Deltaproteobacteria related to the *Desulfocapsa*/*Desulfosarcina*/ *Desulfofustis* cluster.

Specificity of phylotype-specific probes

Based on 16S rRNA gene sequence data from the years 2007 and 2010 (Scott, 2007; Wilbanks *et al*., 2010) specific probes for the two dominating phylotypes in the pink berries were generated. Their specificity was tested *in silico* searching for full-match sequences in the public databases. As these could not be found, probes were

optimized experimentally for their formamide concentrations during hybridization with the target organism. Therefore, berry slices were hybridized with 10, 20, 30, 40, 45 and 50% formamide in the hybridization buffer.

For probe SRB-PiBe213, strong fluorescent signals of the rod-shaped organisms associated with the purple sulfur bacteria could be obtained at 10-45% formamide. Signals at 50% formamide were not distinguishable from background autofluorescence of the aggregate (Figure 2). These results were obtained independent of using methanol/H_2O_2 or HCl as inactivation step. Assuming that the rod-shaped dominant mophotype, which is associated with the clusters of purple sulfur bacteria in the berry-aggregate, is the source organism for the dominant *Desulfofustis*-related sequences, the hybridization results indicate that the probe binds to its full-match target organism at 45% formamide, but not anymore at 50%. This assumption has to be confirmed in future hybridization experiments with a one-mismatch organism, which should not result in any signal when hybridized at 45% formamide.

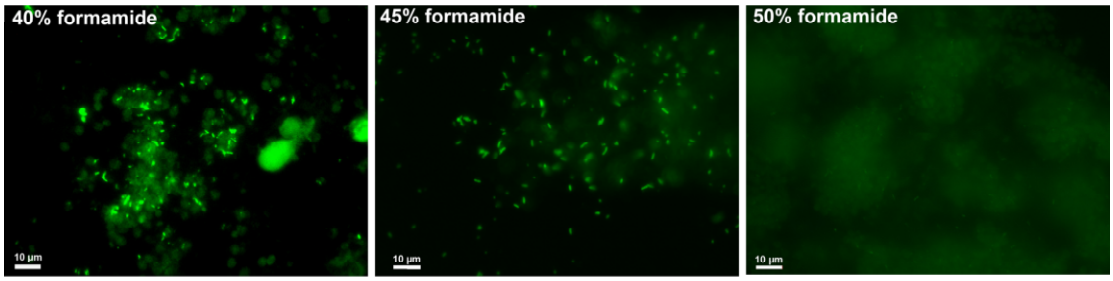


Figure 2. Specificity-experiment of probe SRB-PiBe213. Strong fluorescent signals for dominant, ros-shaped morphotype of berry-aggregate was detected at 10-45% formamide (40% and 45% shown here). At 50% formamide, only the faint background signal of berry-biomass was visible and rod-shaped morphotypes could no longer be distinguished from larger, spherical morphotypes.

Probe PSB-PiBe461, which was designed to target the purple sulfur bacteria-related phylotype in the berry-aggregates, did not show any specific signal for the large, round and pigmented morphotype that dominates the biomass of the berry (Figure 3 A). Signal intensity was about the same at 10-50% formamide and was not distinguishable from background autofluorescence. This result was obtained independent of using methanol/H_2O_2 or HCl as inactivation step. Using the probe mix EUBI-III as positive control clearly showed that the CARD-FISH protocol was designed well for staining also this dominant morphotypes of the berries (Figure 3 B). The failure in specifically hybridizing the *Halochromatium*-related phylotype in the

aggregate therefore leads to the conclusion that either the probe targets a poorly accessible region with in the ribosome of the target organisms, which could be tested in the future by adding helpers to the hybridization buffer (Fuchs *et al.*, 2000), or the main phylotype as revealed by 16S rRNA gene sequencing is not correlating to the main morphotype forming the aggregate.

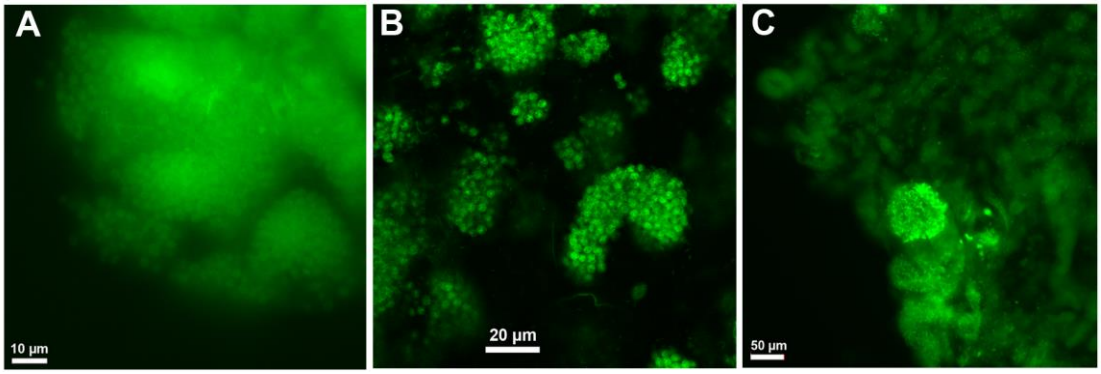


Figure 3. A) Background signal of berry-biomass when hybridized with probe PSB-PiBe461 at 10% formamide concentration. None of the cells in the biomass were significantly brighter than others, suggesting that the probe did not bind to any organisms in the sample, even at such low formamide concentrations. B) Hybidization of berry-slices with the probe mix EUBI-III shows that the CARD-FISH methods is suitable to stain the large, spherical cells forming the main biomass of the berry, which was originally targeted by probe PSB-PiBe461. C) In a less magnified view of berry slices hybridized with probe SRB-PiBe213 patches of dense population/aggregation of *Desulfofustis*-related bacteria become visible.

While hybridizing berry-slices for probe optimization, it was observed that aggregates tend to contain patches of biomass that show a higher density of *Desulfofustis*-related bacteria as revealed by their specific staining (Figure 3C). Sometimes, these densely populated areas were round and in a close-up view corresponded to large clusters of purple sulfur bacteria that were densely surrounded by the stained rod-shaped bacteria. In other aggregate slices, these highly aggregated areas are not round, but the entire rim of the respective aggregate shows more dense population of rod-shaped bacteria. It remains unclear, why there are certain regions in the entire aggregate that tend to be populated in higher densities by *Desulfofustis*-like bacteria than others. Reasons for stronger signal intensities could be a higher concentration of ribosomes in these bacteria, indicating a more active metabolism due to the fact that the outermost cells of an aggregate are not diffusion-limited for nutrients. The patchy, circular distribution of more densely populated areas could be explained by a possible recent attachment of that particular aggregate to the periphery

of the larger aggregate. Possibly, smaller, younger aggregates may be populated more densely with *Desulfofustis*-like bacteria, as discussed below.

Detection of free-living berry-associated phylotypes in pool water and early aggregate-stages in sand

Using the optimized, specific probe SRB-PiBe213 for the detection of berry-associated *Desulfofustis*/*Desulfocapsa*/*Desulfosarcina*-phylotypes, water samples of berry-pools and pink sand from pool-surrounding tide-ways were hybridized. The attempt was to identify the berry-associated phylotype in a free-living stage or in association with the purple sulfur bacteria, but in an early stage of aggregation. Thereby, the putative origin of berry-formation and the possible source of the partners from free-living stages could be detected. It was suggested previously that berries form in the sand banks and are transported to the pools via the tides (Seitz *et al*. 1993).

Analysis of pink sand samples showed a large number of aggregates stained with DAPI (roughly 20-50 µm in diameter), consisting of the typical large, spherical berry-associated morphotype identified as the purple sulfur bacteria. Interestingly, only few of the many aggregates show aggregation with *Desulfofustis*-related rod-shaped bacteria as it can be found in the large aggregates from the pools (Figure 4A-C). The non-populated sulfur bacteria-aggregates seem to be either populated by rod-shaped bacteria that do not stain with the specific probe (but visible with DAPI) or do not seem to be associated with other bacteria.

The hybridization of the pool water revealed specifically stained single rod-shaped bacteria in very low amounts (Figure 4D). Compared to total cells stained with DAPI they make up by estimation <1%. The analyzed filter were too densely covered with cells and organic material, which did not allow a thorough counting to get actual numbers. Also, putative beginning-stages of berries were detected. Figure 4E shows a small aggregate of six large, spherical cells, possibly purple sulfur bacteria as those typically found in large berries, and two rod-shaped bacteria, which are specifically stained with the probe for *Desulfofustis*-related organisms usually found in large berries.

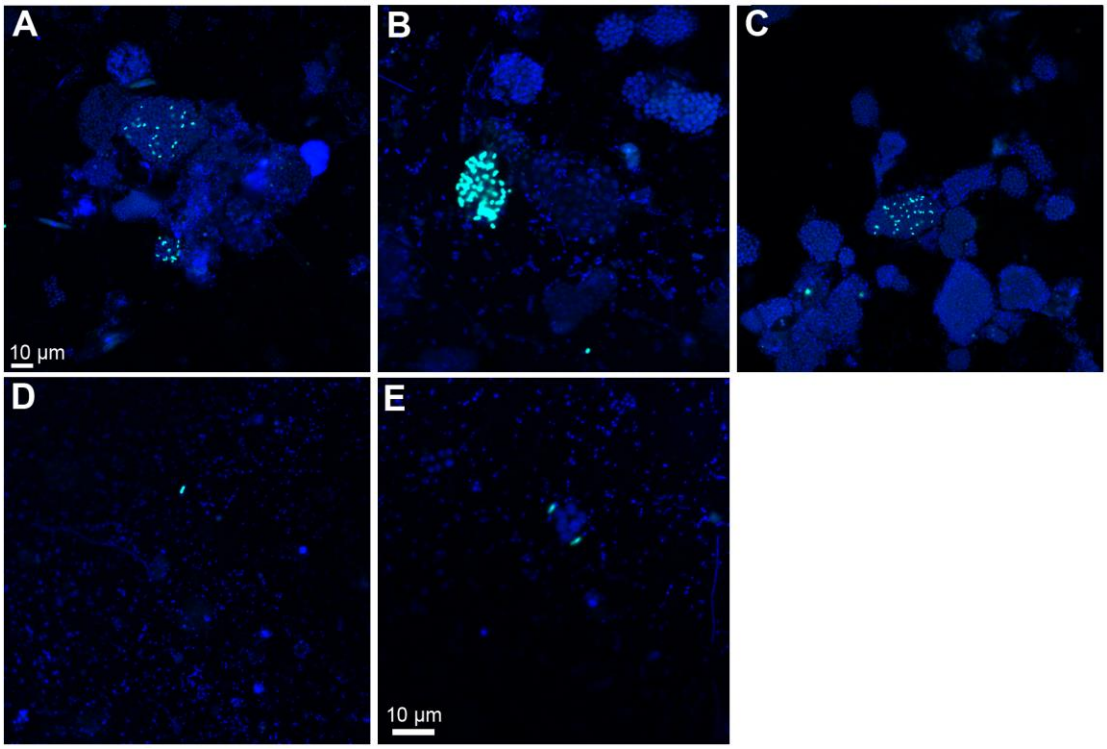


Figure 4. A-C) CARD-FISH with probe SRB-PiBe213 and DAPI-stain of pink sand from tide-ways separate from berry-pools in Little and Great Sippewissett salt marsh. DAPI-stain reveals the presence of a large number of small aggregates reminiscent of clusters forming the large berries found in the pools. Interestingly, only some of the many aggregates are populated by the *Dessulfofustis*-phylotype found regularly in large berries. The other aggregates seem to be either populated by other rod-shaped or bacteria or are not populated.

D, E) CARD-FISH and DAPI-stain with probe SRB-PiBe213 of water overlying berry-pools. Few free-living rod-shaped bacteria are visible and occasionally a small aggregate (like in E) was detected, consisting of the larger, round purple sulfur bacteria and the rod-shaped *Desulfofustis*-like bacterium.

These findings show that there do exist free-living stages of the *Desulfofustis*-like bacteria as known from the large berries in the overlying water, however, their relative cell numbers seem to be fairly low. Also early stages of berry formation can be observed in the water of the pools, indicating that berries form from free-living bacteria and as the aggregate grows larger it possibly sinks down into the sediment of the pool. The detection of large amounts of these smaller berry-aggregates in pink sand in tide-ways physically well separated from the berry pools suggests that this may indeed the main location of aggregate-formation. During high tide, small aggregates are likely suspended into the water column and are transported across the entire marsh area. When aggregates are physically moved like this they may tend to attach to each other forming larger aggregates, as demonstrated earlier (Seitz *et al.*

1993), and when reaching a critical size they my sink down into the pools and accumulate there. Considering this putative random aggregation of different small aggregates forming larger constructions, it may serve as explanation for the finding of a patchy distribution of *Desulfofustis*-related bacteria in the overall biomass of a large aggregate. This assumption is based on the observation that when small aggregates from the pink sand were populated with the *Desulfofustis*-phylotype, they were mostly observed to be very densely populated (Figure 4B). It remains open though, with which phylotypes (possibly other Deltaproteobacteria) the other small aggregates of purple sulfur bacteria are associated, or whether they are not associated, yet, with other bacteria.

Arrangement of purple sulfur bacteria and their associated Desulfofustis-*like bacteria in clusters*

In an earlier study performed in the microbial diversity course (Wilbanks *et al*. 2010), berry slices were hybridized with Gamma- and Deltaproteobacteria-specific probes, which revealed the arrangement of the main berry-biomass in patches or clusters. These clusters were found to be dominated by the two well distinguishable morphotypes of large, spherical cells and small rod-shaped cells. The latter populated the outside and the small areas between the large spheres within the cluster. Due to the lack of phylotype-specific probes, however, it could only be speculated that these two morphotypes and their physical association correlates with the dominant phylotypes identified in clone libraries. In this study, however, the newly designed probe specific for the *Desulfofustis*-related phylotype was used and did not only confirm the presence of this phylotype as the main rod-shaped bacterium within the aggregate, but it could also be shown that this phylotype is the other main morphotype forming the biomass-cluster besides the spherical purple sulfur bacteria (Figure 5). Some of the cluster-forming morphotypes are also found in the interstitial space between the biomass-patches, which could on the one hand be a true observation, but could on the other hand be also be an artefact due to the shearing forces during cryo-sectioning.

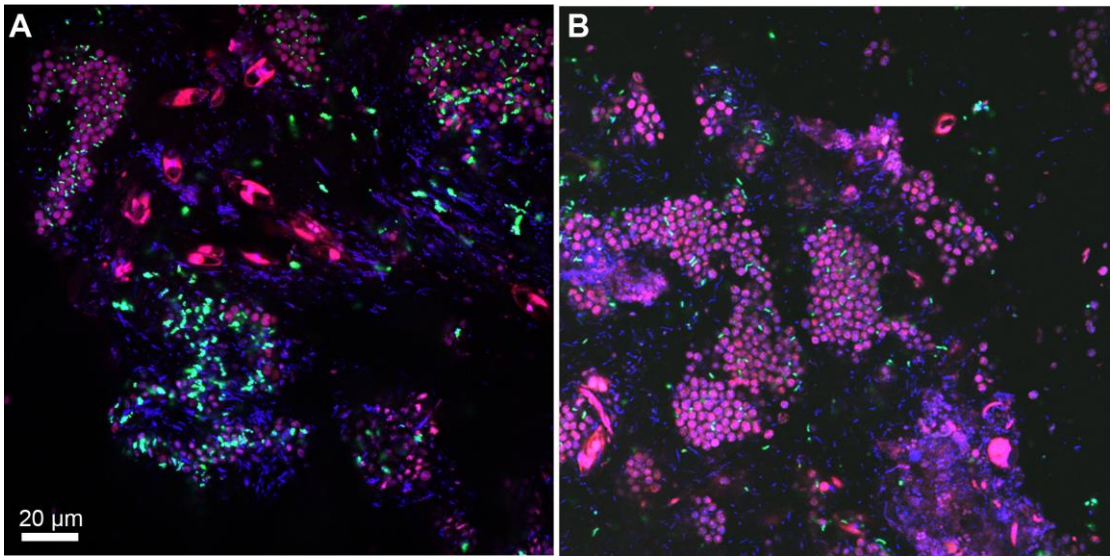


Figure 5. Image of a berry-slice taken with the confocal laser scanning microscope. The sample was stained with DAPI (blue) and the *Desulfofustis*-specific probe SRB-PiBe213 (green) and the autofluorescence of the purple sulfur bacteria was recorded in red. The distribution of the main biomass in the berries becomes visible as patches or clusters formed by the two main morphotypes of large, spherical purple sulfur bacteria and smaller rod-shaped *Desulfofustis*-like bacteria. In between these clusters DAPI-signals reveal the presence of multiple other bacteria with a varying morphology, but only few of the patch-forming morphotypes are found here.

Cultivation of sulfate reducers from berries

Due to the $FeSO_4$ in the culture medium, grows of sulfate reducers can be detected according to the formation of FeS, which a metallic black color. Accordingly, in shake tubes the formation of black colonies could be detected and in liquid medium the precipitation of FeS was observed.

The first two tubes of each dilution series turned completely black within 12 hours. Two days after inoculation, several single black colonies were observed in the glycolate-supplemented shake tube of dilution 10^{-3}. Four days after inoculation, few black colonies were also visible in acetate- and lactate- supplemented tubes (dilution 10^{-5}). Two colonies of each of the latter tubes were recovered and streaked onto agar plates. Another two were recovered and inoculated into liquid medium. From the glycolate-tube, 10 colonies were streaked onto plates and four colonies were inoculated into liquid medium. As the H_2/CO_2-tubes did not show any colonies, yet, 0.1 mL of the liquid culture were streaked on plates and incubated in an aerobic jar flushed with H_2/CO_2. The plates did not show any growth of colonies within the next 10 days until the end of the course. Some of the plates turned black (the H_2-plates

turned black within a day), but the source of this reaction could not be localized. Assuming that the change in color of the plates is caused by the activity of sulfate-reducing bacteria, their biomass seems to be extremely low and colonies for isolation are not visible.

The liquid cultures, on the other hand, all showed a change in color within hours after inoculation. The H_2/CO_2-liquid culture even turned black within 1 hour. Also those liquid cultures of smaller volume (30 mL) that were inoculated with a single colony from the shake tube turned black within a day – the H_2-bottle changed color again with 1 hour. From the liquid cultures, 0.1 mL were streaked onto agar plates, but no colony growth was visible until the end of the course, as described above. Microscopic examination of the liquid enrichment cultures showed a high morphological diversity of cells, most of all, however, containing motile rods, curved rods (vibroid) and spirilla. The liquid cultures resulting from single colonies of the shake tubes showed a more homogenous picture and generally only a single morphotype was visible per bottle. Mostly, the cells were motile rods or slightly bent rods (vibroid). In order to test, whether the cultured sulfate-reducers are the *Desulfofustis*-related phylotype of the berry-aggregate, which shows aggregation with the purple sulfur bacteria, the specific probe SRB-PiBe213 was tested on filtered culture samples. However, no signal could be obtained, which means that the single-colony cultures did not contain the main putative sulfate-reducing phylotype of the berries.

However, the immediate color change in liquid enrichment cultures and the first dilution stages of the shake tubes demonstrates that sulfate-reducers are highly abundant in the berry-homogenate. These sulfate-reducing bacteria seem to be extremely efficient in sulfide production when having H_2 as their electron donor. Glycolate was tested as electron donor because it was hypothesized that this compound might be a shared intermediate between the purple sulfur bacteria and the putative sulfate-reducers as both their closest relatives are known to use or excrete glycolate (*Halochromatium glycolicum* and *Desulfofustis glycolicus*). At last in the glycolate-added shake tubes, a difference to the other conditions was observed. Single colonies were visible earlier than in the other conditions, however, they grew already in the 10^{-3} dilution. This means that there were not as many cells present in the inoculum that used this compound as electron donor, which contradicting the hypothesis that the putative sulfate-reducing phylotype in the berry uses glycolate as

electron donor. On the other hand, as all organisms recovered from the shake tubes seem to not be the main phylotype indentified in the berry previously it can still not be excluded that they use glycolate. At the same time, it cannot be proven that this main phylotype is reducing sulfate and may be causing the rapid color change in liquid enrichment cultures. The *Desulfofustis*-related phylotype is highly abundant in the berry and could therefore very much cause the sulfide production in liquid culture, but might be poorly adapted in agar culture. Even though being the most abundant sulfate-reducing organism in the inoculum, it might well be that when diluted into shake tubes, this phylotype does not grow and other sulfate-reducing phylotypes present in the homogenate in lower cell numbers grow in the tubes.

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