A Microscopic Investigation of Purple Sulfur Bacterial Aggregates From Sippewissett Marsh

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Abstract.
Purple berries, macroscopic aggregates of purple sulfur bacteria, are found in tidal pools of Sippewissett Salt Marsh. They have been observed and researched for about a decade, primarily by the MBL Microbial Diversity course. Over the past several years of the course, Microbial Diversity researchers have characterized the physiology and species composition of the berries. Previous researchers suggested that purple sulfur bacteria (PSBs), Cytophaga, and sulfate-reducing bacteria (SRBs) are major components of the berries. In this project, microscopy was used to test those hypotheses. Here, results from microscopy are shown. Results suggest that the berries are primarily purple sulfur bacteria as expected, but there does not seem to be a consistent presence of Cytophaga in the berries. SRB FISH was inconclusive and needs to be repeated. Microscopy also shows that PSBs cluster in distinct microscopic formations within the purple berries, and they exclude other microorganisms including cyanobacteria that are found throughout the berries. FISH experiments showed that most of the cells in the aggregates belong to the γ -proteobacteria, including both the large purple sulfur bacteria in the clusters, and also other small rod-shaped bacteria surrounding the PSB clusters.

Introduction

Purple "berries" are aggregates found in tidal pools of Sippewissett Marsh, and they have caught researchers' attention for at least the past decade. However, there remains much to be understood about what they are, their ecological significance, and how they form. Seitz *et al.* first characterized purple berries in the literature in 1995. Based on morphology, it was determined that the berries are aggregates of *Chromatiaceae*, purple sulfur bacteria (PSB). Purple pigment of the cells is visible under phase microscopy, and intracellular sulfur granules are visible under darkfield microscopy. Analysis of extracted pigments by Seitz *et al.* confirmed the presence of bacteriochlorophyll a, further confirming the presence of purple sulfur bacteria. Seitz *et al.* stated in their paper that the berries were primarily made of purple sulfur bacteria (PSBs). However, they also noted the presence of other bacteria, including short stubby rods. In 1997, Udi Banin did a mini-project on the berries with the goals of further characterization of the microbial communities in a purple berry. In this project, cloning and sequencing yielded sequences from PSBs, Cytophaga, sulfate-reducers and two sequences most closely related to a sulfur-oxidizing endosymbiont and an"unidentified marine clone." Because the Cytophaga and SRB sequences dominated in numbers in the clone library, it was inferred that those species might be important in the purple berry community. In addition, Cytophaga and SRB sequences were found across all berries sampled. In addition to these DNA results, culture-based results showed that SRBs and Cytophaga could both be cultured from the berries, along with other anaerobic phototrophs (Banin, 1997; Bedard, 1998). These data were interpreted to suggest that berries might be aggregates of PSBs, Cytophaga, and SRBs, and that these species interacted in a complex micro-food web where SRBs produce sulfide, "feeding" the PSBs.

Although it is interesting to make conjectures about possible species-species interactions within bacterial aggregates, these results only suggest that these species are present within the aggregates. They do not imply abundance or significance of any of the species represented by the sequences/cultures obtained in the laboratory. In order to understand the makeup of a berry, visualization of the microscopic structure of various berries is necessary. In this project, epifluorescence and confocal laser scanning microscopy were used in conjunction with cytological stains and specific oligonucleotide probes to elucidate the architecture of purple berries on a microscopic scale. In addition, some experiments were done to understand the effects of different DNA isolation procedures on sequence diversity.

Materials and Methods

Collection

"Berries" were collected with water and sediment from the tidal pools in Sippewissett Marsh and brought back to the laboratory where they were kept in sealed jars in the pool sediment and water. Berries from different pools were kept in different containers to ensure that a wide range of berry types and lineages were observed. Berries were immediately rinsed and fixed for microscopy upon return to lab. When fresh tissue was photographed, unless otherwise noted, berries were used fresh (no more than 1 day old) for microscopy.

DNA extraction protocols

Some methods were attempted to extract PSB DNA from the berries, but PCR results suggest that although amplifiable bacterial DNA was extracted from the berries, there was either very little or no PSB DNA in the samples. It was noted that the PSB

clusters were difficult to dissociate, so several protocols were used in an attempt to break them up, and the results were assessed using light microscopy. Bead beating with the beads from the Mol Bio Soil DNA Extraction Kit and with 1mm glass beads were both tried for periods from 5-10minutes. 0.5g berry was bead-beated in lysis buffer (150mM NaCl, 100mM EDTA, 10mg/ml lysozyme). In addition, the berries were incubated in 200uM urea for 3 hours, then put in lysis buffer and bead-beated. In another experiment, 1g purple berry was frozen and pulverized in a frozen (-80°C) ceramic mortar and pestle, then incubated in lysis buffer at 37°C for 1 hour for extraction with Qiagen DNEasy DNA extraction. This extraction procedure seemed to break up the PSB cells the most and was used for PCR experiments.

PCR

To test if the DNA extraction was successful, DNA was run out on a 0.8% agarose gel and run at 70V for 45 minutes. Universal eubacterial 16S rRNA primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') were used with Taq DNA polymerase on a BioRad iCycler with the following conditions: 1x(95°C 5 min), 35x (95°C 30sec, 45-65°C gradient 30 sec, 72°C 1 min), 1x(72°C 7 min). Also used were the primer sets targeting the photosynthetic *puf* genes *pufM*557f (5'-CGCACCTGGACTGGAC-3')/*pufM*750r (5'-CCCATSGTCCAGCGCCAGAA-3'), and *pufL*f(5'-CTKTTCGACTTGTGGGTSGG-3')/*pufM*750r. In addition, a 16S rRNA primer set targeting specifically purple sulfur bacteria was used: PSB 16S 25F (5'-AGAGTTTGATCMTGGCTC) and PURPR (5'-CAGCGTCAGTGTTGAACC-3' (T. Marsh design).

Microscopy

Berries were cut equatorially and stained with SybrGreen (10^{-5} x stock) for 10 minutes at RT. These were examined under the Zeiss Axioscope with the Chroma "FITC" filter set. In addition, it was informative to visualize the berries under the "RHODAMINE" filter set. These images displayed autofluorescence of chl a, which emits around 660nm. Stained fresh berries were also visualized on a Zeiss Axioscope confocal LSM. Samples were viewed under the "RHOD/FITC" setting, a multi-channel setting saved with the attached confocal image files. Approximate sizing of cell clusters and spacing between clusters was measured using the distance measurement function of the Zeiss LSM software.

Fluorescent In Situ Hybridization (FISH)

For FISH, fresh berries were cut in half, preserved in freshly diluted 4% formaldehyde (in 1X PBS) for 16 hours at 4°C. The samples were then rinsed 2X with 1X PBS and transferred into a 30% sucrose solution as a cryoprotectant. They were in sucrose for at least 12 hours (until the berries sank to the bottom of the tube and were fully infiltrated with sucrose) before cryosectioning. We found that if they were in for more than a few several days before sectioning, they crumbled very easily and were hard to manipulate for sectioning. After the berries were infiltrated with sucrose, they were cryosectioned to 30um sections in a cryotome at –20°C (by highly skilled fellow student Margy Gentile). The sections were placed directly onto gelatine-coated glass slides and stored at –80°C.

To prepare the slides for hybridization, the slides were removed from the freezer ad dried in a 46°C oven. Because of problems with sections falling off of the slides, they were then coated in 0.1% agarose (gel strength 1,200g/cm^2) and dried again at 46°C. For

hybridization with all probes except the β and γ probes, 10ul of hybridization solution (10ul Cy3-labeled probe (50ng/ul) + 20ul hybridization buffer (900mM NaCl, 20mM Tris-HCl, 35% formamide, 0.01% SDS)) was added to the top of each section (2 per slide). In the β and γ probed samples, 10ul of labeled probe, 10ul of non-labeled competitor probe, and 10ul of hybridization solution was added. For example, 10ul of the Cy3-labeled β probe was added and 10ul of the unlabeled competitor γ probe were added to 10ul hybridization buffer. The coated slides were placed in humidity chambers containing hybridization solution and incubated for 5 hours at 46°C. After hybridization, the slides were incubated in wash buffer (900mM NaCl, 20mM Tris-HCl, 5mM EDTA, 0.01% SDS) at 48°C for 30 minutes. The slides were briefly rinsed in QH2O, air-dried at RT, stained with DAPI, and mounted in CitiFluor anti-fade medium.

* note: all of the hybridizations were performed with formamide concentration at 35%. The following rRNA oligonucleotide probes used were all added from stock concentration of 50ng/ul:

Target	**Probe name**
General Eubacterial	EUB338
Alpha subclass of proteobacteria	ALF968
Beta subclass of proteobacteria	BET42a
Gamma subclass of proteobacteria	GAM42a
Cytophaga-Flavobacteria cluster	CF319a
Archaea	ARCH315
Eubacterial non (negative control –one base mismatch)	EUB non

In an attempt to eliminate autofluorescence from the tissues, both freshly fixed berries and fixed cryosections were treated with 7:2 acetone:methanol for 10 minutes at RT. In both samples, the cells were damaged by the treatments, and the autofluorescence was not diminished. This method was not used for the FISH experiments, but an alternative method that successfully extracts or bleaches pigments from the cells would be a great asset to getting robust and repeatable FISH results from berry sections.

Results

DNA Work

There was consistent amplification with the eubacterial primers from DNA extracts, and the 250bp product from the *pufM* primer pair was amplified successfully. However, neither the larger *puf* product pimers nor the PSB-specific 16S primers successfully amplified this DNA extract, 45-65°C annealing temperature gradient. The primers did amplify PSB control DNA across most of the temperature gradient, suggesting that the primers were OK but the DNA extraction was problematic.

Microscopy

Figure 1a is a representative phase contrast image of a piece of a berry. In this image, there are distinctly organized clusters with distinct channels surrounding the clusters. In these channels, other organisms, including photosynthetic eukaryotes and other microorganisms, were noted (not shown). No organisms other than PSBs were observed within the PSB clusters. This is in contrast to Figure 1b, which is a picture of a purple clump from sediment around Oyster Pond. The Oyster Pond purple clump contains distinct clusters of PSBs, but they are much smaller, and do not seem to exclude other organisms to the same scale as the clusters in the purple berries.

Figures 2a,b show images of a purple berry, looking down on a berry cut equatorially. The pattern in the colors of the berries is consistent with and representative of the 80 berries sampled. The berry tissue is light pink on the outside and dark pink/purple in the middle. On closer look, this color difference was correlated with sulfur granules in cells – the lighter layer contained cells with more sulfur granules, while the middle section contained cells that had very few intracellular sulfur granules.

Figure 3a shows an epifluorescence image of a SybrGreen-stained berry. It is striking that there is no difference in cell morphology or arrangement/density of cell clusters across the berry. These patterns seen with the SybrGreen fluorescence matched the patterns seen with a filter set that allowed visualization of autofluorescence emission in the IR spectrum, suggesting that these clusters are PSB clusters. Figure 3b shows a closer look at some of the clusters.

Figure 4 shows an example of autofluorescent filamentous cyanobacteria. Both filamentous and unicellular cyanobacteria are commonly distributed throughout the berries, and there is no apparent pattern to their distribution. In images obtained from confocal microscopy, a matrix that surrounded PSB clusters was apparent, and it seemed to exclude other bacterial morphotypes. In addition, there was distinct spatial separation between cyanobacteria and the PSB clusters. According to the measurement tool in the Zeiss LSM software, cyanobacterial "islands" ranged anywhere from 30um wide to 150um wide, while PSB clusters could be equally as large. There were always at least 10um between PSB clusters, and on average at least 80um between PSB clusters and cyanobacterial islands.

Figure 5a shows a close-up image of a Sybr-Green stained PSB cluster. Note that around the cluster are many smaller, rod-shaped bacteria. In FISH, shown in Figure 5b, these cells hybridize to a γ–proteobacteria probe, along with the PSB in the clusters.

Conclusions

Structure and Species Composition of Berries

These results suggest that berries are conserved aggregates containing PSBs, cyanos, and some small unicellular γ-proteobacteria. The berries, regardless of size, all consist of highly ordered clusters of PSBs, and these clusters contribute to aggregates that show order on a macroscale as well. The species structure and the spatial structure on a microscopic scale seem to be conserved across all berries and are the same throughout a single berry. It should be noted that the color change across the diameter of a berry does not reflect a different species, but a difference in concentration of intracellular sulfur granules in the PSB clusters. This suggests that different coloration in berries may be related to the metabolic state of the bacteria within, rather than the species composition or pigment synthesis. These results are slightly in contrast to the conclusions drawn from previous research. Previous research efforts concentrated on culture-based analyses and isolations of specific bacterial groups from berries. It was possible to culture all anaerobic phototrophs from the berries, and also Cytophaga and SRBs. These groups of bacteria were then a focus of the purple berry research, but it was still not know how significant a role these bacteria played in the formation or maintenance of the berries. The in situ microscopy analysis was very important in determining what cell

morphotypes are consistently major constituents of the purple berries. It also should be noted the clone library from this year showed only 1 PSB sequence out of 100 sequences, while other marine bacteria were present in the clone library. This probably is a product of incomplete cell lysis in the DNA extraction, which was problematic in these experiments as well. Without microscopy, it is impossible to distinguish between bacteria that are truly major components of the system and bacteria that are part of the tidal pool water column and were incidentally associated with the sampled berry. It is not surprising that Cytophaga, a common and widespread group of marine microorganisms, could be detected and cultured from purple berry inoculum. It is also not surprising to find sulfate-reducers from sampling that included anoxic marsh sediment in the tidal pool. It was my original hypothesis that SRBs produced sulfide and presented a "nucleating center" for the PSBs to attach and start building an aggregate. However, the majority of the cells in the berries seem to be accounted for with the microscopy. The clusters are PSBs, and the small unicellular rods surrounding them seem to belong to the γ-proteobacteria as well. Cytophaga probes did not light up any cells in the berries. It is necessary to do some FISH experiments with probes targeting SRBs determine the presence and distribution of SRBs in the berries. The results of this year's experimentation suggest that sequences thought to represent major constituents of the berries may actually be incidental marine microorganisms that came in with sampling. This emphasizes the importance of confirming molecular data with microscopy. According to microscopy, the berries seem to be made primarily of PSBs, cyanobacteria, and at least one species of small unicellular γ-proteobacteria. Identification of the gammas surrounding the PSB clusters could elucidate their role in the berry – the high level of organization in the berries suggests that they may play a specific role in the aggregates. It also will prove useful to do an exhaustive search with SRB-specific FISH

probes, to see if they occur in the berries and if they are at all localized to a certain region.

Method Development: modification of FISH protocol for thicker sections

There were a few changes in the FISH protocol that proved to be very important for getting this protocol to work. Conventional cryosectioning methods were used. However, the sections would not stay on the glass slides by themselves, whether or not they were gelatine-coated. Before using the cryosections on gelatine-coated slides in FISH, the slides were coated again by dipping into 0.1% high gelling strength agarose and dried at 46°C for about 20 minutes. This proved to keep the sections attached throughout the hybridization and washes, and it also allowed the probes to diffuse into the sample. There were a couple of adaptations to adjust for the thickness of the sections (30um). The probe concentration was increased from 50ng/ sample 250ng/sample. In addition, the hybridization time was increased from 2 hours to 5 hours, and subsequently the washing incubation time increased from 20 minutes to 30 minutes. These changes were adapted from an original protocol from S. Behrens for FISH on seawater filtrate on polycarbonate membranes. This adapted protocol is shown to work well for thick sections (30um) that do not stick to the glass slides.

Origin of Berries/Future Directions

The results from the class report by Castro *et al.* suggest that purple sulfur bacteria are expelled as pseudofeces by mussels and may be what they called "immature berries." Atlantic mussels were observed expelling tiny clusters of PSBs in the field in

1998. However, this year in the field, pools that contained berries but did not seem to contain mussels were observed. This is not a conclusive argument against the involvement of mussels in berry formation, as the berries may spill over from pool to pool, or from pool to mat, or from mat to pool. This "spillover" phenomenon was observed this year in the field: outside the boundary of a berry-containing pool were many small berries in the top layer of the sediment. This calls for future research exploring the origin of the berries. One aspect to explore is the origin of the extracellular slime matrix that holds the clusters together. Is it of eukaryotic or bacterial origin? Castro *et al.* suggested that the mucus that holds them together arises from the mussels. This needs to be tested using cytological stains, or an array of specific lectins. Results from Seitz *et al.* suggest that the matrix is made of polysaccharides, but the origin remains unknown. It would also be interesting to design some experiments to pursue growth and development of berries in laboratory mesocosms. In addition, since they contain at least two types of photoautotrophic microorganisms, experiments characterizing responses to changing light regimes in species composition, metabolism or physiology of the berries could elucidate the metabolic processes and physiology of the aggregates as a whole. Comparative studies with other free-living, planktonic purple sulfur bacteria would also provide insight into the aggregates. This calls for the identification of the purple sulfur bacteria in the aggregates. Microscopy and morphology suggest that they are Chromatiaceae, but they should be identified with molecular data.

Literature Cited

Banin, U. Microbial Diversity Mini-Project Report, 1997.

Bedard, D.L. Microbial Diversity Mini-Project Report, 1999.

Castro, H., Fujita, Y., Patterson, K., and E. Zinser. Microbial Diversity Mini-Project Report, 1998.

Seitz, A.P., Nielsen, T.H., and J. Overmann. 1995. Physiology of purple sulfur bacteria forming macroscopic aggregates in Great Sippewissett Salt Marsh, Massachusetts. *FEMS Microbiol. Ecol.* 12:225-236.

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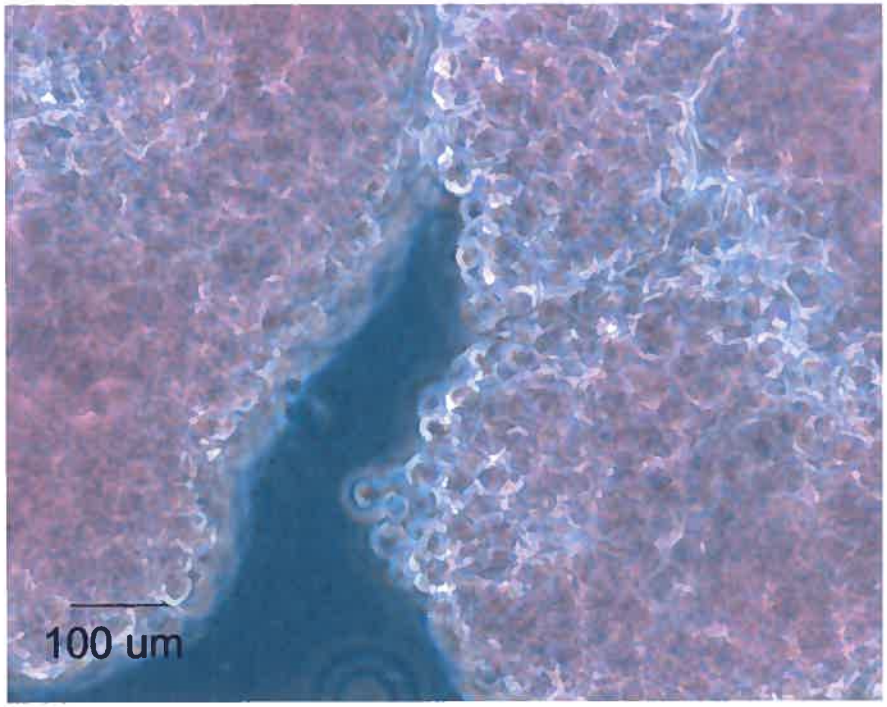
Purple Berry



Oyster Pond Sediment

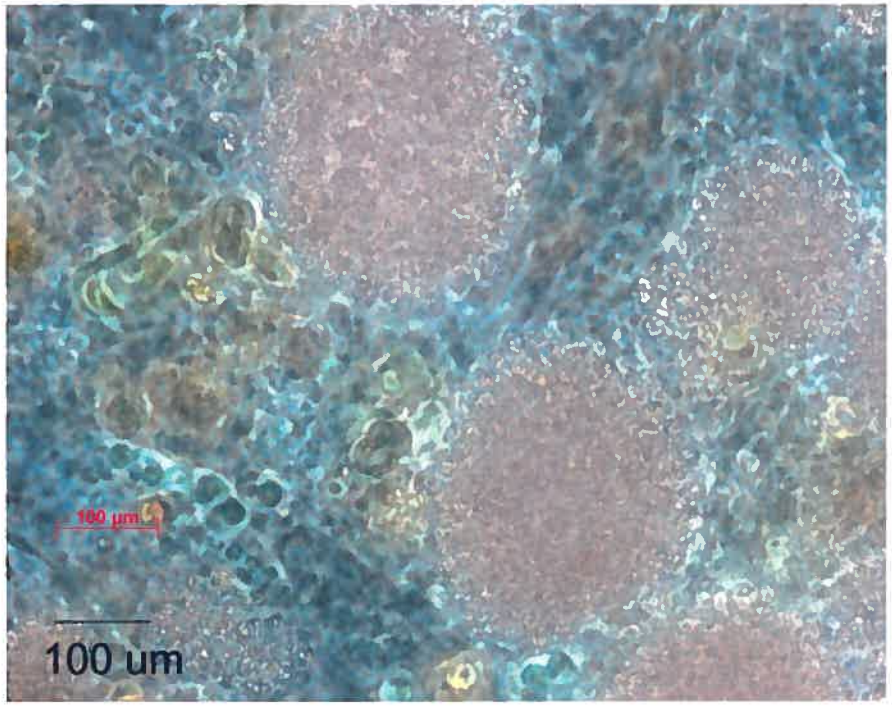


Figure 1. (a) shows clusters of purple sulfur bacteria tightly associated with each other. (b) shows a purple clump from sediment that was not as organized and contained many other types of organisms.

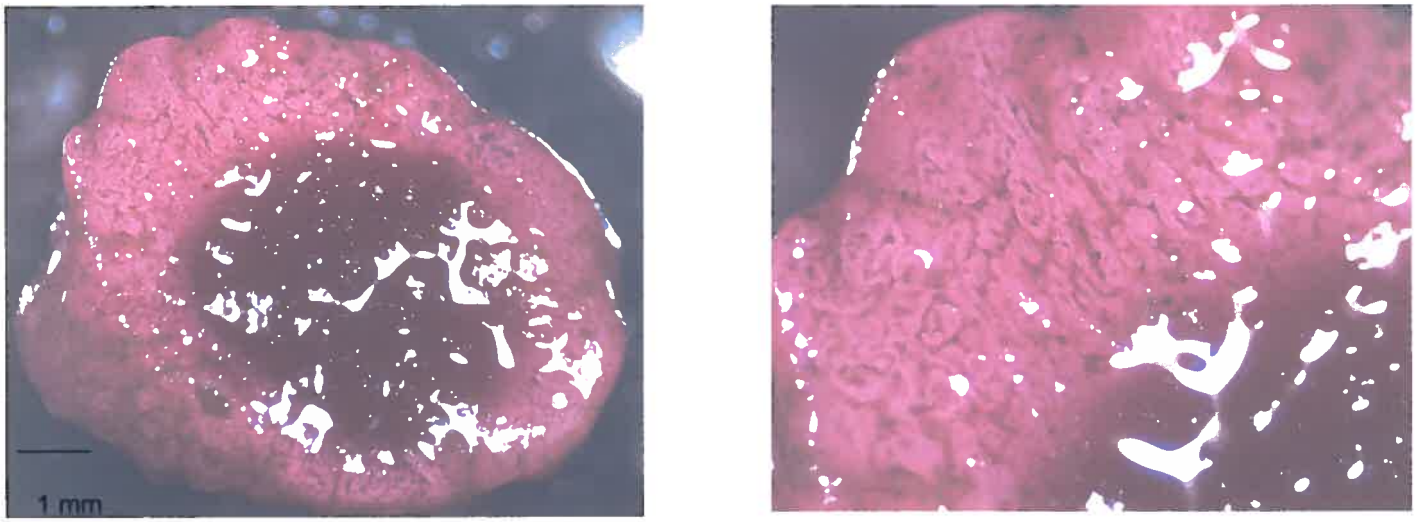
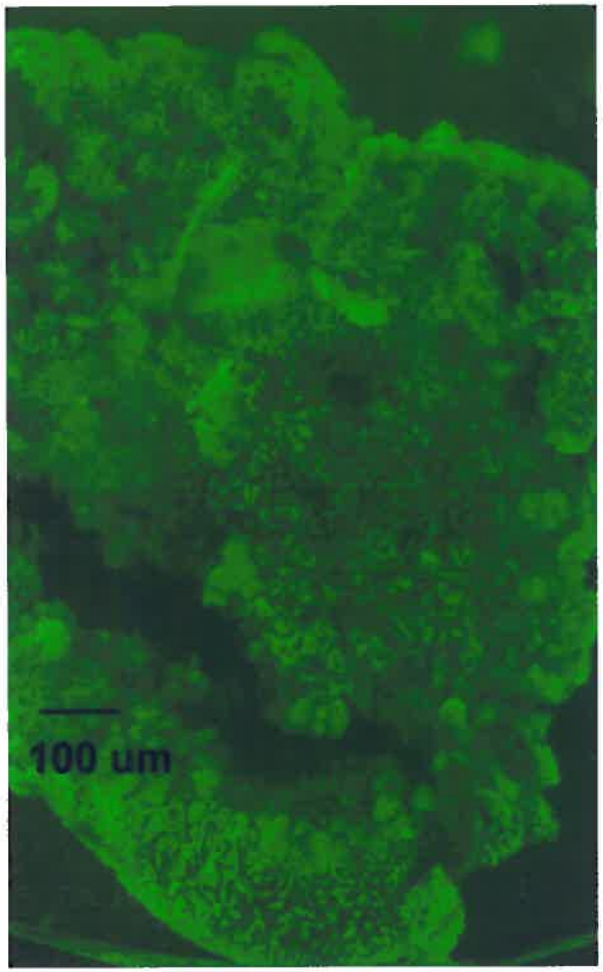


Figure 2. (a) shows a look down across the middle of an equatorially cute purple berry. (b) shows a closeup of the light purple section containing distinct cell cluster patterns.



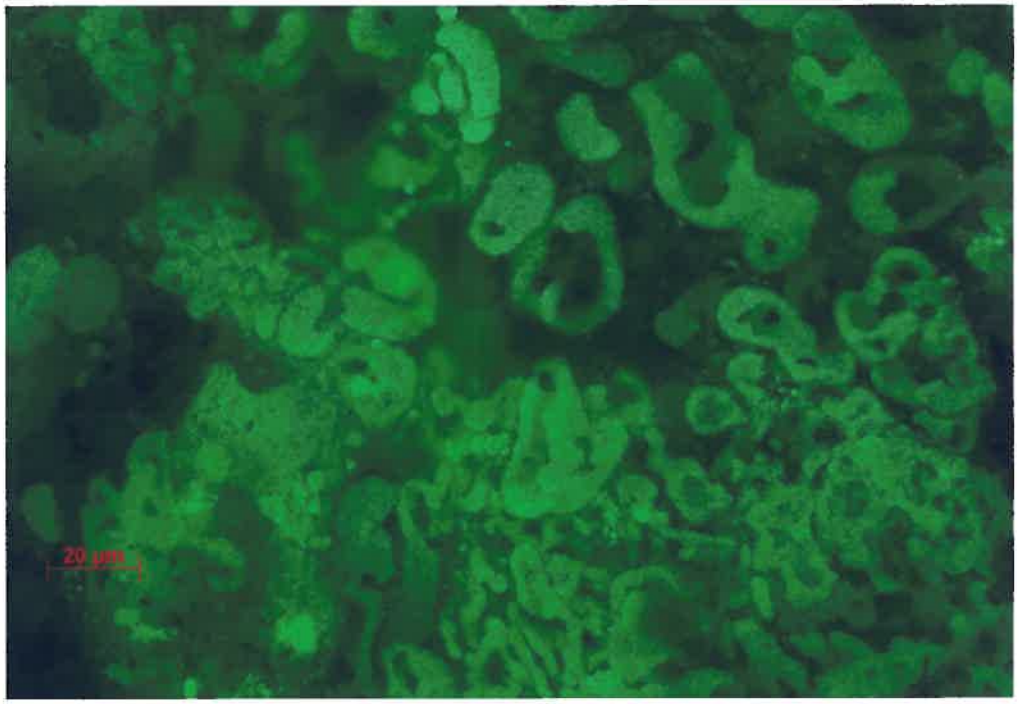


Figure 3. SybrGreen-labelled purple berries. 3(a) shows a view of a the surface of an equatorially cut bacteria. 3(b) shows the organized clusters from within the berry.

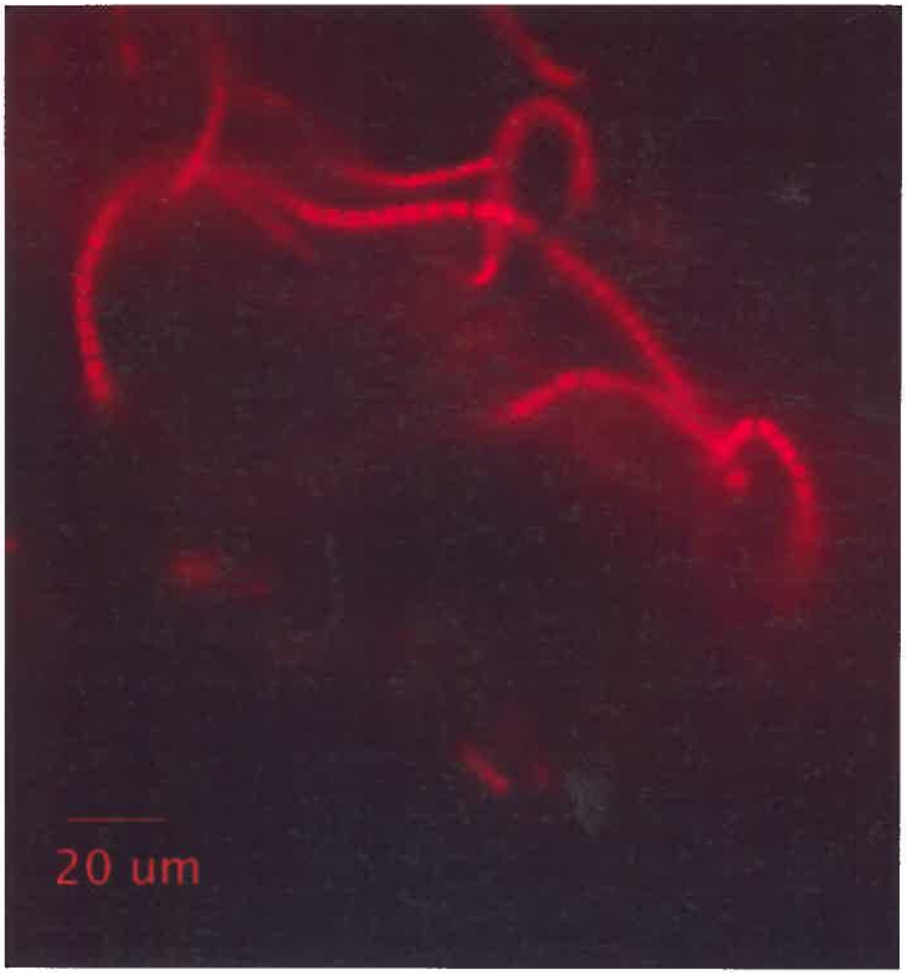


Figure 4. Cyanobacterial filaments in a cluster within the purple berries. They are localized separately of other purple sulfur bacteria in the berry. Note the obvious heterocysts (non-chla containing cells).

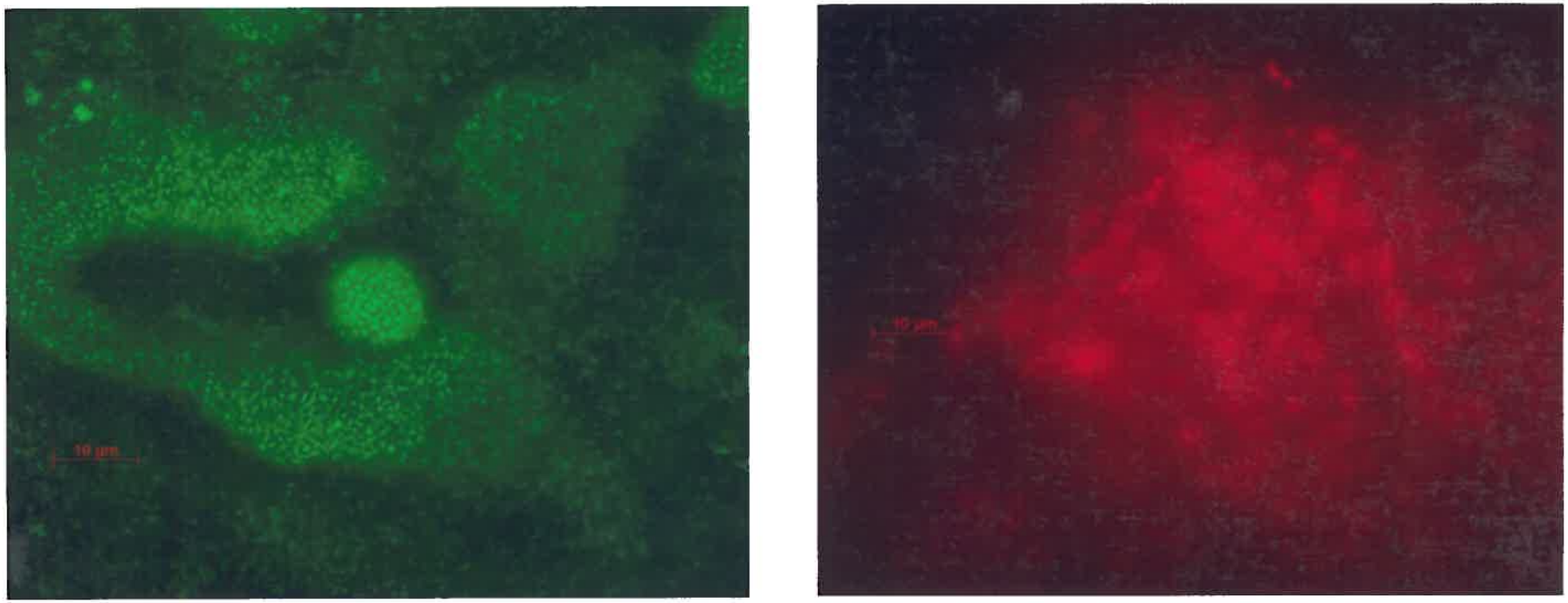


Figure 5. 5(a) shows a high-magnification image of SybrGreen-stained berry tissue, focusing on the clusters of purple sulfur bacteria. Surrounding the clusters are small rod-shaped bacterial cells. 5(b) is the FISH image with the Cy3-labeled gamma proteobacteria rRNA probe. The probe hybridizes to the PSBs as well as the small unicellular bacteria around the cluster.

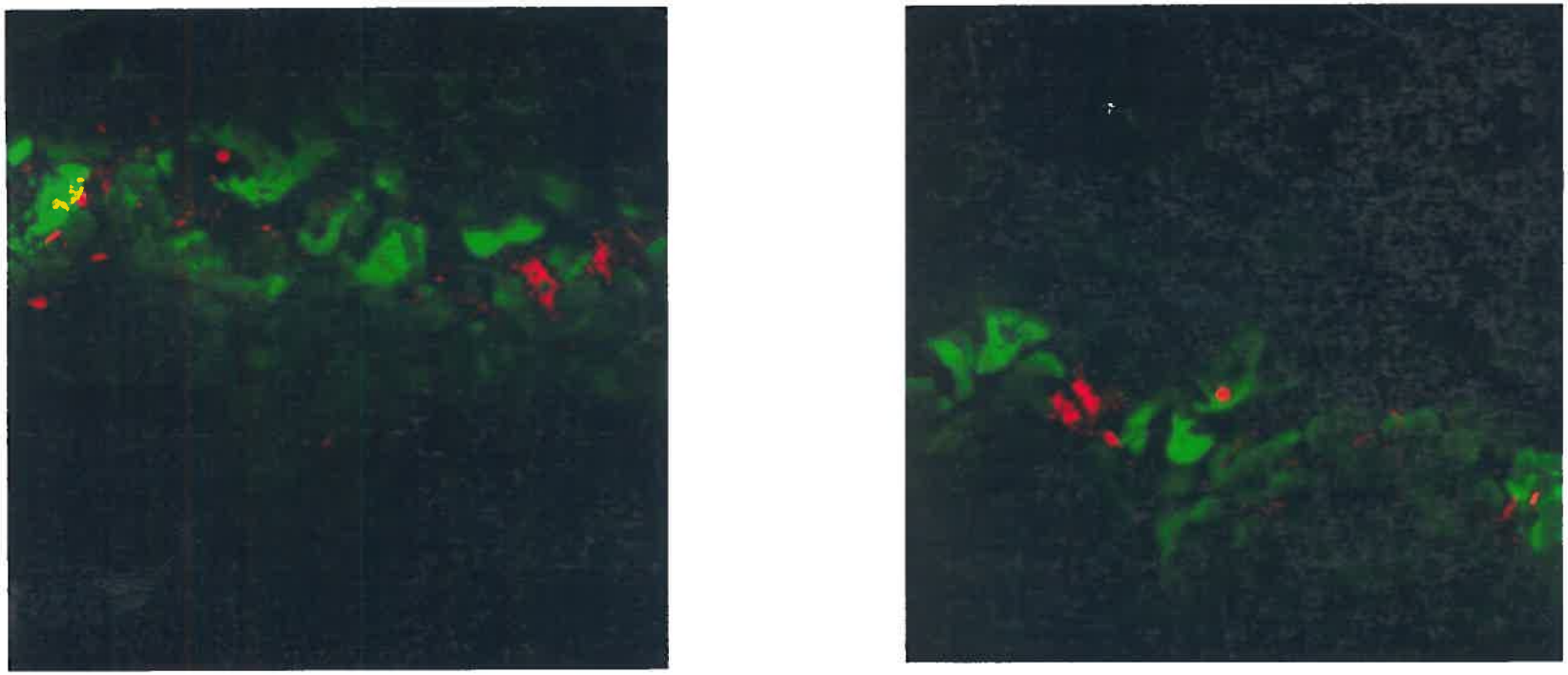


Figure 6. 3-dimensional images from the laser scanning confocal microscope. The images show evidence of a matrix surrounding the clusters of purple sulfur bacteria and excluding other bacteria, most markedly the cyanobacteria. There is much more information in the full z-stack files (on attached CD, and the documents should be viewed with Zeiss LSM5 Image Browser (available on the internet).