BACTERIAL DIVERSITY IN "BERRIES" COLLECTED FROM TIDAL POOLS ON THE GREAT SIPPEWISSETT SALT MARSH

DONNA L. BEDARD

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Bacterial Diversity in "Berries" Collected from Tidal Pools on the Great Sippewissett Salt Marsh

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The "berries" that can be found in some of the tidal pools on the Great Sippewissett Salt Marsh are macroscopic microbial communities dominated by purple sulfur phototrophic bacteria 1, 3, 15). Purple sulfur phototrophs are photosynthetic anaerobes that use light as an energy source and sulfide as an electron donor. They oxidize the sulfide to sulfur which is often accumulated intracellularly and ultimately to sulfate (7, 11, 12). The berries are apparently formed and expelled by mussels feeding on "pink sand" in the marshes. Previous studies have identified some of the microbial species in the berries by a combination of traditional enrichment and isolation, and molecular techniques (See references 1, 3, and 15).

As part of our group project, we used a homogenate of washed berries to inoculate three different series of anaerobic phototroph enrichments on agar shakes. From the wide range of different colonies that formed in these enrichments it was apparent that the microbial composition of the berries is far more diverse than previously thought. My objectives were to recover a wide variety of microscopically pure colonies from these phototrophic enrichments, to characterize their colony and cell morphology, and to use molecular sequence analysis to identify them on the basis of their 16S rRNA genes.
METHODS

Phototrophic Enrichments

Berries were collected from tidal pools on the Great Sippewissett Salt Marsh and stored in sea water at 4°C in the light until used. To prepare an inoculum, berries were washed several times in sterile sea water and then homogenized with a Dounce homogenizer. Two sets of agar shakes prepared with each of three different phototroph enrichment media were inoculated with six serial dilutions (seven-fold each) of the berry homogenate inoculum under anoxic conditions using the Hungate technique. The enrichment conditions used were for purple sulfur phototrophs, green sulfur phototrophs, and non-sulfur phototrophs. All media were prepared for enrichment of phototrophs from brackish habitats according as described (5). Briefly, we used agar shakes for spatial separation of phototrophic colonies.

The medium for the "non-sulfur" phototrophs was adjusted to pH 6.8 - 6.9 and included succinic acid, glutaric acid, malic acid, and acetate (each at 5 mM) as electron donors. The base medium also contained a total of 7 mM sulfate [from the MgSO₄ and (NH₄)₂SO₄]. The inoculated shakes were sealed with a layer of paraffin wax to ensure anaerobicity and incubated at room temperature using a 12h:12h dark:light cycle. Light was supplied from a 40 watt incandescent bulb approximately 30-40 cm from the shakes.

The medium for the phototrophic "sulfur" bacteria included oxygen-free NaHCO₃ (20 mM) as a buffer, and carbon source, and acetate and pyruvate (each at 1.5 mM) as supplementary carbon sources. Sulfide (Na₂S) was provided as the electron donor. The enrichment for Chromatiaceae (purple sulfur phototrophs) was adjusted to pH 7.2-7.4 and contained 1 mM Na₂S. The enrichment for Chlorobiaceae (green sulfur phototrophs) was adjusted to pH 6.8 -
6.9 and contained 2.5 mM Na₂S plus 5 mM thiosulfate (Na₂S₂O₃). These shakes were also sealed with paraffin wax and incubated as described above.

**Colony Recovery**

Colonies in the shakes were examined using a dissecting microscope (Zeiss Stemi SV II or Zeiss Stemi 2000-C, 10X objective plus 0.65 - 5X additional magnification) illuminated from above with halogen lamps. Colonies exhibiting different morphologies were noted and selected for recovery and characterization. Colony characteristics were described and in some cases photographed. Digital images were captured and saved on a computer using the MetaMorph Imaging System. The paraffin sealing the agar was melted over a small flame and poured out, then the agar shakes were extruded from the incubation tubes into a sterile Petri dish by inserting a 25 μl capillary to the bottom along the wall of the tube and blowing. Sterile fine jewelers forceps were used to separate regions of the shake containing single colonies. The agar containing the selected colony was then transferred to a second sterile Petri dish and most of the agar surrounding the colony was removed. Each isolated colony was given a letter designation and then divided into two or three parts. Whenever possible, a wetmount slide was immediately prepared from part of the colony, examined microscopically under phase contrast, and photographed. Two portions of each colony were transferred to 200 μl Eppendorf tubes: one was stored at -20°C for future sequencing; the second was stored at 4°C for further microscopic examination. A total of 26 colonies were recovered and examined microscopically.

**Microscopic Examination of Bacterial Cells**

Bacterial cells from the isolated colonies on wetmount slides were examined under phase contrast using a Zeiss MC 80DX microscope equipped with 10X, 20X, 40X, and 100X
objectives, 10X eye pieces, and a 1 to 2.5X magnification feature. Digital images were captured and saved on a computer using the MetaMorph Imaging System. Images were calibrated and stamped with a 5 to 10 μm bar to show the size of the cells.

**DNA Extraction, PCR Amplification, and Sequencing of 16S rRNA Genes**

DNA was extracted from the colonies with PrepMan. The colony was suspended in 300 μl of sterile cross-linked UltraPure water and transferred to a sterile 1.5 ml microfuge tube. The cells were centrifuged for 3 minutes at 9,000 rpm. The supernatant was carefully removed with a pasteur pipet taking care not to resuspend the pellet. The cells were resuspended in 200 μl of PrepMan solution by pipetting. (PrepMan solution contains detergent to lyse the cells and a chelex resin to bind and precipitate the proteins). The cells were incubated at 56°C for at least 30 minutes, then vortexed for 10 seconds and heated in a boiling water bath for 8 minutes. The tubes were centrifuged for 2 minutes at 15,000 rpm. The clear supernatant was transferred to a clean sterile tube and diluted 1:10 with sterile cross-linked water. For most colonies I recovered 50 μl of clear supernatant. Five μl of the pure DNA was amplified by PCR using Eubacterial primers for the 16S rRNA gene. (The forward primer was a 20 nucleotides beginning at E. coli position 8. The reverse primer was 19 nucleotides long beginning at E. coli position 1492). The polymerase was PFU (Stratagene). The PCR thermal cycle conditions were: Initial denaturation: 94°C for 12 minutes, followed by 30 cycles of amplification: Denaturation of the template, 94°C for 1.5 min, Annealing of primers, 60°C for 45 seconds, extension, 72°C for 1 minute. The final extension was at 72°C for 3 minutes.

The PCR-amplified product from all colonies except those that were obviously not pure were given to the course instructors to be sequenced. However, due to time constraints only 6
colonies were sent out to Forsyth Labs for sequencing. These were the PCR products from colonies G, J, R, V, X, and Z. PCR product from colonies P, Q, Y, W, and T were to be sequenced in house.

RESULTS

Colony Diversity

Figure 1 shows some of the diverse colony types observed in the enrichments. Colonies X and Y were both observed in the purple sulfur phototroph enrichments. Both of these colonies were pure white and were clearly not phototrophs. To the naked eye both of these colonies looked fuzzy and could easily be mistaken for fungi. Closer examination with the dissecting microscope revealed their beauty. Colony X looked like a spherical crystal snowflake. Colony Y was more compact and looked like a snowball. The bottom panel in Figure 1 shows a variety of different colony types in a purple phototroph enrichment tube. These include shiny disc shaped colonies of in various shades of pink, red, and purple, black fuzzy colonies, and a pinkish colony with a white halo. The colonies that appear green in the picture did not appear green in natural light; the color is apparently an artifact of the lighting conditions used for the picture.

Colonies J, F, and A (Figure 1) were present in the green sulfur phototroph enrichment tubes along with other colonies including a few jet black colonies and several pink and purple colonies. Colony J was a very unusual colony that had the appearance of a beautiful white coral. Only two colonies of this type were seen, and only in the tubes with the least inoculum. Colony F was a white colony with a dense center and a fluffy halo. Colony A was a brownish beige shiny disc shaped colony.
Purple sulfur phototroph enrichments.

Colony Q This was a large heart-shaped purple colony. The colony completely dispersed when gently touched with the point of the forceps indicating that the cells were not tightly associated. The individual cells were an ovoid shape, about 2 - 3 μm in size, and each contained several sulfur granules. Figure 2 shows phase contrast images of the cells photographed with white light and no filter (top) or with a blue filter (bottom). The cells are clearly purple sulfur phototrophs.

Colony R This was a large pinkish purple colony which dispersed easily when touched with the forceps. The cells were ovoid, and were 3 - 5 μm in size, but many much larger cells were also present (Figure 3). Some of these were clearly dividing and it is likely that the very large cells that I observed are the result of continued growth without subsequent division. All cells were purple and were filled with sulfur granules. Again, the cells are clearly purple sulfur phototrophs.

Colony S This colony was purple, but was deep red when broken open. The cells were similar to those of colony R, but there were not as many dividing cells (Figure 4). In addition, there were some very dark cells without as many sulfur granules. These cells are also purple sulfur phototrophs.

Colony T This colony was a large purple colony that was easily dispersed. The cells look like those of colony R and many were in the process of dividing (Figure 5). There were many extremely long cells present. Again, these apparently are cells that have continued to grow but are unable to divide. The morphology indicates that these are purple sulfur bacteria.
Colony V  This colony was pinkish with red striations, a bit like chicken liver. The color was much lighter than that of colonies Q - T and the colony was much more solid. It did not disperse easily but had to be cut apart. The cells were tiny round bacteria (~ 1 μm diameter) and each contained 1-3 sulfur granules (Figure 4). These also appear to be purple sulfur phototrophs.

Colony W  This was a whitish beige disc shaped colony. The cells were curved vibrio-like rods, ~ 0.5 X 3 μm (not shown). These are clearly not phototrophs.

Colony X  This was the white snowflake-like colony shown in Figure 1. The cells were small vibrio-shaped cells (~ 0.5 μm diameter and 3 - 5 μm long). They were often associated with large sulfur precipitates (Figure 6). Based on their lack of pigmentation, these do not appear to be phototrophs.

Colony Y  This was the white "snowball" colony shown in Figure 1. This colony contained several different cell types including short highly motile rods (~ 0.3 X 1 μm), curved rods (~ 0.5 X 2 μm), and tiny spherical cells (Not shown).

Colony P  This was a very large amorphous fluffy colony. The cells were motile curved rods, (~ 0.5 X 2 μm) (Figure 7).

Green Sulfur Phototroph Enrichment

Colony N  This was a large colony that looked fuzzy to the naked eye. Examination with a dissecting microscope revealed that the colony had a brownish oblong dense center with a white fuzzy halo. Except for the color, it was similar to colony F (Figure 1). There appeared to
be at least two cell types present: short fat slightly curved rods, and longer spirillum-shaped cells (Figure 8).

**Colony A** This brownish beige disc-shaped colony (Figure 1) was quite solid. The cells were spirillum-like.

**Colony B** This was a purple colony similar to those seen on the purple phototroph enrichments. The cells were round and were full of sulfur granules. They appear to be purple sulfur phototrophs.

**Colony G** This was a jet black round rough colony. The cells were not examined microscopically until after several days of refrigeration. At this point very few cells were found. The cells appeared to be of two types: curved rods that formed a star-shaped cluster and small spheres (Figure 9).

**Colony I** This was a deep crimson amorphous colony with some veils around it. The colony contained several cell types: small spirillum shaped bacteria, ovoid sulfur-filled cells, and a long filament containing sulfur granules (Figure 10).

**Colony J** This was the white coral-like colony shown in Figure 1. The cells were fat slightly curved rods (~ 1 μm diameter by 1.5 - 3 μm long) (Figure 11). Each contained a colorless inclusion.

**Colony K** Without the aid of the dissecting microscope, this appeared to be a single fuzzy white colony, but microscopic examination revealed that there were actually multiple colonies arranged with one small central colony surrounded by many tiny satellite colonies in different orbits. The cells were motile curved rods swarming around large masses that appeared to be tightly packed cells (Figure 10, center panels).
Colony L  This was a large red colony that was slightly fuzzy, not smooth. The cells are vibrio-like, ∼ 3 µm long, and are very active. From the tracks captured on the image (Fig. 10, lower left panel) they were moving at ∼ 750 µm per second! The refractile spherical bodies on the image appear to be an artifact of the cell contact with the coverslip, not inclusions or spores.

Colony M  This was a large pink colony. Microscopic examination showed that there were several cell types present. Some were tiny sulfur-filled bacteria (similar to those of colony AA, Figure 12). Others were small rods and spheres.

Purple Non-sulfur Phototroph Enrichments

Colony AA  This was a small reddish purple colony. It was quite dense and looked like a tiny berry when viewed with the microscope. The cells looked like tiny sulfur granules and could only be clearly identified as cells when they were present in dense clusters (Figure 12). The bottom panel of the figure also shows a curved rod.

Colony Z  This colony had a dark brownish black opaque center but the outer portion was translucent and light coffee colored. The colony was loose and easily dispersed. The cells were slightly curved rods, most of which were packed together in large dense clusters (Figure 13).

Sequence Results, Phylogenetic Mapping, and Tentative Identification.

Sequences for colonies G and R failed, but I received sequences (about 230 nucleotides long) for colonies J, V, X, and Z. These were initially aligned automatically using the ARB program (9, 10). The alignment was then refined manually on the basis of comparison with sequences from related bacteria. The trees obtained for 16S rRNA genes from these four colonies are shown on Figures 14 - 17. No sequences were obtained for any of the remaining colonies.
DISCUSSION

The phototrophic enrichments yielded a wide variety of colonies and cells. Based on microscopic appearance and the inclusion of sulfur granules, there were at least three types of purple sulfur phototrophs. The first type had large ovoid cells filled with sulfur granules and contained some long cells that appeared to result from growth without division. Examples of this were colonies R, S, and T. These cells most likely belong to the genus Chromatium. The second type had smaller round cells with several sulfur granules. This type is illustrated by colonies Q and V. The third type was very tiny and looked like individual sulfur granules. These cell membranes could be seen only when the cells were clumped together (Colony AA). Even though this latter colony was obtained from the “non-sulfur” enrichment, the enrichment did in fact contain sulfate and a sulfate reducing sequence was identified in these enrichments (see below). Thus sulfate-reducing bacteria could have produced sulfide and lowered the redox potential sufficiently to allow growth of at least a few purple sulfur phototrophs.

The findings reported here for three types of purple sulfur phototrophs in the berries are consistent with previous studies (1, 3) and our own observations (15) which showed that the dominant cell types in the berries were purple sulfur phototrophs. One previous study (1) identified a Rhabdothermatium sp. from 16S rRNA genes amplified by PCR from berry DNA and cloned in E. coli. A single Rhabdothermatium species has been described. This species has rod to spindle-shaped cells that are 1.5 to 1.7 µm wide and 16 to 32 µm long (11). None of the colonies were composed entirely of cells that fit this description, but it is possible that some of the very large cells in colonies R and T might be Rhabdothermatium. Sequence information would be needed to confirm this hypothesis. A subsequent report of a nearly identical cloned sequence from berries (1 base pair difference out of 500) indicated that the closest relative was
*Thiorhodococcus minus*, not *Rhabdothermatium* (3). *Thiorhodococcus minus* was recently isolated from a coastal lagoon on the Atlantic coast of France. It was described as spherical to ovoid cells 1 - 2 µm in diameter which multiply by binary fission, accumulate sulfur granules, and occurs as single cells or aggregates. These characteristics are similar to what we observed, but *T. minus* is highly motile and none of the purple sulfur phototrophs that I received are motile. Since the 16S rRNA cloned from the berry was only 94% similar to *T. minus*, it is likely that the purple sulfur phototrophs in the berries belong to different species and perhaps even different genera than have previously been described. Unfortunately, I did not receive sequences for any of the purple sulfur phototrophs so it is not yet possible to link sequences to the different phenotypes observed. The sequence obtained from colony V was that of a *Pseudomonas* sp. related to *P. aeruginosa* and was apparently a contaminant. This was unexpected because microscopic examination revealed only the purple sulfur phototrophs.

Even though there were many different colony types in the green sulfur phototroph enrichments, I did not see any green colonies or any cells that appeared to be green sulfur phototrophs. This finding is consistent with our checkerboard hybridization of the DNA extracted from berries which failed to hybridize with a probe from green sulfur bacteria (15).

From the variety of non-pigmented, beige, brown, and black colonies present, it is apparent that there are also many non-phototrophs present in the berries. Indeed, cells from colonies J, X, and Z were identified by 16S rRNA sequence analysis as a *Propionigenium* sp., a *Vibrio* sp., and a sulfate reducing bacterium, respectively.

The 16S rRNA sequence from Colony J was 96% similar to that of *Propionigenium maris*, and 92% similar to that of *Propionigenium modestum*. These are the only species of this genus that have been described. This genus is strictly anaerobic and can grow by decarboxylation of
succinate to propionate (8, 13). The two species reported were both isolated from marine environments and appear to require 100 to 150 mM concentrations of sodium ions as coupling ions for energy conservation (8, 13). Growth on pyruvate is poor. These characteristics are consistent with the conditions in the green sulfur phototrophic enrichments from which these colonies were isolated. *P. maris* cells were described as being oval to round-ended rods, usually 1 μm X 1.2 to 2.5 μm. This is similar to the morphology of cells from colony J, but no inclusions were described for either of the reported *Propionigenium* species and the cells of colony J clearly have inclusions (Figure 11).

The 16S rRNA sequence from Colony X clearly fell within the *Vibrio* genus and was most similar to *Vibrio nigripulchritudo* (97%). This species was originally described as *Beneckea nigripulchritudo* (2) and was later reclassified as *Vibrio* when the genus *Beneckea* was dropped. Most of the *Vibrios* are marine organisms, but most are aerobic. Another related species, *Vibrio gazogenes*, is a sodium dependent facultative anaerobe (6), and a probe from this organism did hybridize to berry DNA on the checkerboard analysis. But *V. gazogenes* has a bright red pigment, and colony X was white. *V. nigripulchritudo* differs from colony J most conspicuously by its color which is due to extracellular deposits of a blue black insoluble pigment (2) (hence its name "dark beauty"). *V. nigripulchritudo* apparently was not tested for the ability to grow anaerobically.

The 16S sequence from Colony Z clusters within the sulfate reducing bacteria but a genus could not be assigned from the sequence data. Its nearest neighbors appear to be *Desulfospira joergensenii* and various species of *Desulfobacter*. The Desulfobacter are usually oval, but are sometimes curved rods as is sp. Z1 (14). The *Desulfobacter* are typically brackish or marine organisms and require NaCl at 100 mM and MgCl₂ at 5 mM for optimal growth (14). Acetate is the most common and characteristic electron donor of these bacteria (14). It is ironic
that the sulfate reducing bacteria was identified from a "non-sulfur" phototroph enrichment, but the enrichment medium actually contained 7 mM sulfate and 5 mM acetate and thus could easily support the growth of these bacteria.

Previous hybridization data and cloning data have also indicated that sulfate reducing organisms are present in the berries (1, 3, ). Sulfate reducers are logical inhabitants of the berries and can likely live in a symbiotic relationship with the purple sulfur phototrophs. The sulfate reducers would reduce the sulfate to sulfide which can then be used as an electron donor by the purple sulfur phototrophs. The purple sulfur phototrophs would oxidize the sulfide back to sulfate to act as an electron acceptor for the sulfate reducers. The purple sulfur bacteria may also supply organic carbon to the sulfate reducers. Hence it is likely that some of the unidentified colonies from the purple sulfur phototroph enrichments were also sulfate reducers.

It is quite disappointing that time constraints prevented the successful sequencing of 16S rRNA from any of the colonies that appeared to be purple sulfur phototrophs. Nevertheless, the photomicrographs from the enrichments show that there are at least three phenotypically different types of purple sulfur phototrophs in the berries. Ideally one would pick colonies and use these to inoculate secondary enrichments. This is not possible within the time constraints of this course because it took 4 weeks for the colonies to grow on the enrichments. Indeed, I was able to do this project only because we inoculated the phototroph enrichments during the first week of the course as part of our group project. Hence, the approach taken here still seems like a reasonable approach for obtaining tentative identifications for the purple sulfur phototrophs in future Microbial Diversity Courses. Thermal gradient PCR amplification to optimize the annealing temperature for each DNA template would probably provide better PCR products for sequencing analysis and would promote the chances of success.
ACKNOWLEDGEMENTS

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REFERENCES


**Figure 1**

- **Propionigenium sp. J**
- **Vibrio sp. X**
- **Colony Y**
- **Colonies F and A**
- **Purple sulfur phototroph enrichment**
Colony Q

*Figure 2*
Colony V

Colony S

Figure 4
Colony N

Colony P

Figures 7 & 8
Colony G

Figure 9
Colony I

Colony K

Colony L

Figure 10
Colony AA

Figure 12
Sulfate reducing bacterial species Z1

Figure 13