# Observations and Discoveries Among the Purple Nonsulfur Bacteria

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# Introduction

How does the microbial population of a particular place come to be? It may be that the microbes in a place are primarily derived from local ancestral populations, that adapt (if necessary) to changing conditions or to various microenvironments that exist at a site. Alternatively, it can be imagined that microbes easily disperse from elsewhere to occupy a site, either because they already possess the traits that make them particularly fit in that site, or because there are not strong competitive pressures to exclude new arrivals. The relative importance of local adaptation and dispersal may vary, of course, depending on the organisms, the sites, and the spatial scales investigated.

The purple nonsulfur (PNS) bacteria are an eco-physiological group of anaerobic anoxygenic phototrophs that live primarily as photoheterotrophs; i.e., they generate ATP via photosynthesis and assimilate organic molecules (particularly fermentation products) as a carbon source. However, many PNS are metabolically versatile – capable of photoautotrophic growth with hydrogen or reduced sulfur compounds as electron donors, as well as chemoheterotrophic growth via respiration or fermentation. Such metabolic versatility means that a single species within the PNS bacteria may be expected to be capable of growth in many different environments, although it may not be a superior competitor except in illuminated environments with abundant organic material. In fact, species such as *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides* are known to be distributed widely in aquatic or moist habitats. Such organisms would be particularly appropriate for investigating the relative importance of dispersal and local adaptation.

Oda et al. (Appl. Environ. Micro. , 2002, 68:3467) have investigated the genetic relatedness of *R. palustris* isolates from two estuaries in the Netherlands, one pristine and the other impacted by pollution. Using REP-PCR, a sensitive method of genetic fingerprinting, they found that over a hundred isolates from each site clustered into several groups, but that the groups identified were specific to each site. However, with the exception of growth on benzoate, no physiological characterization of the isolates was done, so it is unclear whether the evolutionarily distinct populations found in the two estuaries reflects adaptation to local conditions or some other phenomenon such as dispersal limitation and drift. This study did establish, however, that it is possible to investigate the spatial structure of relatedness within a widely distributed, metabolically versatile bacterial species.

I imagined that I could perform a similar comparison within a species of PNS bacteria by isolating organisms from the vicinity of Woods Hole and Martha's Vineyard. In addition to evolutionary relatedness, I wanted to investigate whether the organisms found at a site showed adaptation specific to the conditions of that site. I chose to focus on two sorts of environmental conditions. On a larger scale, I planned to isolate organisms from a number of fresh, brackish, and marine aquatic environments separated by either a few kilometers (within Woods Hole or Martha's Vineyard) or by tens of kilometers (between the mainland and the island). Dispersal limitations could possibly affect microbial distributions on this scale. On a smaller scale, within a single sampling site (e.g., Salt

Pond, Falmouth, a brackish site), I planned to isolate organisms from the nearshore water column, the surface of the sediment beneath nearshore water, and from the surface of the soil close to the water's edge. I did not imagine any dispersal barriers existed between the three locations within a site.

In reality, this plan was much too ambitious, even considering the fact that I was prepared to do much of the physiological characterization of isolates once I returned to my home institution. Despite beginning direct isolations of PNS bacteria early in the course, I did not obtain a collection of isolates that would allow me to investigate the question described above. As an alternative, I intended to look for evidence of evolutionary trade-offs in fitness with different modes of bacterial metabolism, by measuring growth rates of isolates under photoheterotrophic and photoautotrophic conditions. Although this may have been a more reasonable goal within the time frame of the course, as it turns out 3 different technical problems (an unknown media composition problem, flocculent growth, and the probable presence of at least two different PNS strains within my 'isolates') renders the growth rate data suspect.

While making some reference to the biogeography investigations I had hoped to carry out (as outlined above), this report will serve mainly to chronicle my experience in trying to isolate and culture a number of PNS bacteria from a range of local habitats.

# Materials and Methods

### Media and Culture Conditions

Direct plating from soil samples from Sippewisset Salt Marsh and School Street Marsh was done by students of the course on PNS media with either ethanol or succinate as a carbon source. Isolation efforts continued on the same solid media.

PNS Medium, per liter:

| | |
|---|---|
| NaCl | 10.0 g |
| $MgCl_2$-$6H_2O$ | 0.4 |
| $CaCl_2$-2 $H_2O$ | 0.1 |
| $KH_2PO_4$ | 0.2 |
| KCl | 0.5 |
| HEPES | 2.38 |
| yeast extract | 1.0 |
| carbon source | 10 mM |
| SL12 trace elements | 1.0 ml |
| vitamin mix | 1.0 ml |

Direct plating from other locations and all liquid media was based on Imhoff's AT medium (The Prokaryotes online edition, Springer-Verlag 2002), which consists of the following, per liter:

| | |
|---|---|
| $KH_2PO_4$ | 1.0 g |
| $MgCl_2$-$6H_2O$ | 0.5 |
| $CaCl_2$-2 $H_2O$ | 0.1 |
| $Na_2SO_4$ | 0.7 |
| $NH_4Cl$ | 1.0 |
| $NaHCO_3$ | 3.0 |
| Na-acetate | 1.0 |
| SLA trace elements | 1.0 ml |
| vitamins | 1.0 ml each stock thiamin, niacin, biotin, PABA at concentrations in vitamin mix. (I mixed the vitamins separately to have the option of adding them individually, although I never did.) |

NaCl added at 1 g/l for Fresh AT (FAT), 10 g/l for Brackish AT (BAT), 30 g/l for Marine AT (MAT).

10 ml of 5% Na-ascorbate added immediately prior to pH adjustment, filtration, and dispensing (or autoclaving) medium to reduce oxygen in the medium. pH adjusted to 6.9, medium autoclaved (solid media) or filter sterilized and dispensed into sterile tubes.

Solid media contained 15 g/l of agar, washed 3x in distilled water; plates were incubated in GasPak jars or in the glovebox with 80% $N_2$/20% $H_2$. Constant illumination was from the side of the plates, either from unshielded incandescent bulbs near the GasPak jars or from an array of bulbs with parabolic reflectors mounted outside the glove box.

For photoheterotrophic growth, 15 mm x 125 mm screwcap tubes were filled completely. Tubes were incubated vertically in large (8x18 position) metal racks, staggered with only half the spaces in the rack occupied by tubes. Constant illumination was proved by 4 40-watt incandescent bulbs in clip-on lamp fixtures with metal parabolic reflectors. Lamps were mounted approximately 15 cm above and several cm to the outside of a square formed by two of the large metal racks. Lights were angled similarly to provide what appeared by eye to be fairly uniform illumination over the area of the racks, and tubes were rotated through the racks regularly to provide uniform average illumination.

For photoautotrophic growth, acetate was omitted, $NaHCO_3$ was increased to 4.7 g/l, 6 ml medium was dispensed to Balch tubes and the headspace filled with 80% $H_2$/20% $CO_2$. Tubes were incubated horizontally in two rows on a large tray attached to an orbital shaker under the constant illumination of 2 40 watt bulbs (without parabolic reflectors) approx 10 cm above the midline between the rows of tubes. Tube positions on the tray were rotated regularly to provide uniform average illumination.

Temperature for photoheterotrophic and photoautotrophic tubes was maintained near 30 C as a result of heat from the lights.

Vitamin mix, per 100 ml:

| | |
|---|---|
| Biotin | 10 mg |
| Niacin | 35 |

| | |
|---|---|
| Thiamine$Cl_2$ | 30 |
| PABA | 20 |
| Pyridoxolium HCL | 10 |
| Ca-panthothenate | 10 |
| B12 | 5 |

Trace Elements SLA, per liter

| | |
|---|---|
| $FeCl_2$-4$H_2O$ | 1800 |
| $CoCl_2$-6$H_2O$ | 250 |
| $NiCl_2$-6$H_2O$ | 10 |
| $CuCl_2$-2$H_2O$ | 10 |
| $MnCl_2$-4$H_2O$ | 70 |
| $ZnCl_2$ | 100 |
| $H_3BO_3$ | 500 |
| $Na_2MoO_4$-2$H_2O$ | 30 |

Dissolve salts separately in water totaling 900 ml, mix solutions, adjust pH to 2-3 with 1 N HCl, adjust final volume to 1 liter.

[Future work: Jane Gibson suggests that for direct plating omitting NH4 doesn't really slow down the PNS bacteria very much, while providing some additional discrimination against many heterotrophs.]

## Sampling Sites

Sampling locations were:

| Area | Category | Site | Isolate code |
|---|---|---|---|
| Martha's Vineyard | Fresh | roadside pond | |
| Martha's Vineyard | Fresh | Chilmark Pond | MVF-Cx |
| Martha's Vineyard | Brackish | Sengekontacket Pond | |
| Martha's Vineyard | Brackish | Farm Pond | |
| Martha's Vineyard | Marine | Quitsa Pond | MVM-Qx |
| Martha's Vineyard | Marine | Vineyard Haven | |
| Woods Hole | Fresh | School Street Marsh (soil only) | Smxx |
| Woods Hole | Fresh | Quisset Pond | |
| Woods Hole | Brackish | Sippewisset Marsh (soil only) | Spxx |
| Woods Hole | Brackish | Salt Pond | |
| Woods Hole | Marine | Great Pond | WHM-Gx |

The roadside pond on Martha's Vineyard is a stagnant 3 m x 10 m artificial pond with no inflow or outflow on the south side of State Rd., just east of the junction with Lobsterville Rd. near Aquinah. Chilmark Pond (my name) is a 10 m x 15 m artificial impoundment

with inflow and outflow on the north side of State Rd. just west of Beetlebung Corner, Chilmark. Sengekontacket Pond is the large, shallow pond on the east edge of Martha's Vineyard between Oak Bluffs and Edgartown, separated from the ocean by a road and beach. There is at least one direct connection between Sengekontacket Pond and the ocean, however, given the area of the pond, the estimated rate of flow through that opening, the estimated land area that would drain into the pond, and the appearance of the surrounding vegetation, I surmised that the salt concentration would not be at marine levels. Samples were obtained by walking west from the beach road near the south end of Sengekontacket Pond to an 'island' of trees and terrestrial vegetation mostly surrounded by the pond and adjoining salt marsh. Farm Pond is a much smaller pond just south of Oak Bluffs, north of Sengekontacket Pond, without (I think) a direct connection to the ocean, but separated from it at the north end only by the width of the road. Samples were obtained from about the middle of the east shore of the pond by walking west from the beach road through a small grove of trees and across a march (watch for poison ivy). Quitsa Pond is the south end of the convoluted water body that extends south from the ocean across the western point of Martha's Vineyard, almost separating the Gay Head/Aquinah area from the rest of the island. Although there is obviously a marine connection, given the area of the watershed and the distance from the ocean I was uncertain whether it might be better to consider this site as brackish. However, at the time of sampling I could see a considerable incoming tidal current. Since there is no substantial river to deliver fresh water to Quitsa Pond, I decided to treat it as marine. The sampling location was at the public boat ramp on the north side of State Rd. on the west side of the bridge over the south end of the pond. The Vineyard Haven site was the beach just north of the Steamship Authority pier. Quisset Pond is on the east side of Sippewisset Road several miles north of Woods Hole. Salt Pond was sampled on the east side, north of the connection with the ocean, accessed from Surf Drive. Great Pond is a marine inlet east of Falmouth Harbor; it was sampled from the area behind a large hotel just south of the bridge over the connection between Great Pond and the ocean.

## Sampling and Direct Plating

At both Sippewisset Salt Marsh and the School Street Marsh, soil samples were obtained by coring the surface of soil or mat material with the large end of a Pasteur pipette. Material was vortexed in sterile buffer, particles allowed to settle, and 100 ml of the buffer spread on PNS ethanol and PNS succinate plates.

Water samples were obtained from other locations by (gloved) hand in 0.5 liter plastic bottles within arm's reach of the shore, or, if the water was so shallow that such sampling would have disturbed the sediment, after wading out to slightly deeper water. Sediment and soil samples from these locations were collected by scooping surface material into 15 ml centrifuge tubes. Tubes were nearly filled with solid material, the remainder of sediment tubes remaining filled with water and the remainder of soil tubes with air. In cases where plant litter was present on the surface of the sediment, a portion of such material was collected as well. At Chilmark Pond, thick layers of decaying leaves covered the bottom of the pond to such an extent that essentially the entire 'sediment' sample consisted of plant litter.

Samples were collected without temperature control and transported back to the lab as soon as practical. Water samples were filtered through 0.45 μm pore size, 47 mm dia. nylon membrane filters with gentle suction (using a Venturi faucet device) until flow rate slowed considerably; typically about 100 ml of water was filtered though each filter. Two such filters were placed on the surface of a single agar plate; in order to fit the second filter was placed in a sterile petri dish and cut in half using a razor blade. At least two plates were prepared per water sample.

About 20 ml of the water that passed through the 0.45 μm filters was filter sterilized and used for processing sediment and soil samples that were taken from the same location. 15 ml centrifuge tubes were filled approximately half-full with sediment or soil, and the remaining volume of the tube filled with the filter-sterilized water from the site. The tubes were shaken horizontally at 30 C overnight, and particles allowed to settle for a few minutes. 100 μl of overlying water was plated directly. A more concentrated cell sample was also obtained by centrifuging 1.7 ml of the overlying water at 5,000 g for 10 minutes, removing 1.6 ml of supernate, and plating the remaining material after resuspending the pellet.

The 6 (or more) plates with material from a site (water, soil sediment) were of a single salt concentration matched to the category of the source material.

## PCR Amplification

Primers for amplification of the small subunit ribosomal RNA gene (16S) were standard bacterial 8 Forward and 1492 Reverse. Amplification was for 30 cycles at the standard temperature parameters: 95 C denaturing, 55 C annealing, and 72 C extension (30 sec each). Cycles were preceded by 5 min of initial denaturing at 95 C and followed by a 7 min final extension at 72 C.

Primers for amplification of the Internal Transcribed Spacer (ITS) region between the small and large subunit ribosomal RNA genes were designed to match conserved regions at the end of the small subunit (16S) gene, and close to the beginning of the large subunit gene (23S). Design was based on literature reports (Gurtler & Stanisich 1996, Microbiology 142:3, Anthony et al. 2000 J. Clinical Micro. 38:781, Trcek & Teuber 2002, FEMS Micro Letters 208:69) as well as sequences in the RDP database (www.cme.rdp.msu.edu). Although designed for the alpha subgroup of the Proteobacteria, they appear to have perfect or near-perfect homology to many other groups as well -- in fact the forward ITS primer near the end of the 16S rDNA gene targets the same conserved region typically used for as a general bacterial reverse primer for amplifying the 16S gene. Assessing the range of homology for the reverse ITS primer was more difficult due to the relative paucity of 23S gene sequence database.

The ITS primers were:
1491 Foreword: 5'-AAG TCG TAA CAA GGT A-3'
LSU188 Reverse: 5'-CTT AGA TGT TTC AGT TC-3'

PCR amplification of the ITS region was for 30 cycles using the same parameters mentioned above, except for an annealing temperature of 41 C. [Future work: A slightly higher annealing temperature, perhaps around 43 C, may be preferable.]

PCR reactions for both 16S and ITS used standard concentrations of reagents in 50 or 100 µl final volume. . Template DNA was obtained by suspending a single medium-sized colony or several tiny colonies of an organism in 10 µl of 1% NP-40 detergent, and subjecting them to 5 cycles of 95 C –4 C, with 1 minute at each temperature. Unless otherwise noted, 1-2 µl of the resulting crude cell lysate was used per PCR reaction. PCR products were separated by standard gel electrophoresis techniques, stained with ethidium bromide, and photographed under UV illumination using the Kodak EDAS gel documentation system.

## Optical Density Measurements and Spectral Analysis

Routine optical density measurements to track growth were made in the growth tubes using a Genesys20 spectrophotometer at 600 nm. Estimates of light harvesting complex proteins (in vivo absorbance peaks 803-805 nm) and bacteriochlorophyll a (in vivo absorbance peaks 849-862 nm) were made in the growth tubes at the actual peak absorbance wavelengths specific for each culture as determined by spectral analysis.

[Future work: For a better estimate of in vivo protein concentrations via culture optical density measurements, it may be worth investigating the utility of subtracting the OD at the wavelength of an absorbance trough adjacent to the peak of interest. The logic is as follows: The total optical density of a culture at the wavelength of peak absorbance of a protein of interest will be the sum of 3 factors: the specific absorbance by the protein of interest, any specific absorbances by other unknown proteins or culture components, and the nonspecific turbidity (scattering) of light due to the presence of cells or other particles. The optical density of the culture at a nearby wavelength corresponding to a trough in absorbance by the protein of interest will mostly be due to the last two factors only. If the optical density due to these factors is approximately equal at the wavelength of the trough and the wavelength of the peak, the trough OD value could be subtracted from the peak OD value to isolate the desired quantity, the specific absorbance of the protein of interest. The assumption of similar contribution to OD by the two confounding factors at two nearby wavelengths is reasonably good in the case of turbidity; it may even be possible to correct for the effect of different wavelengths from the known function of scattering as a function of wavelength. The specific absorbance's due to other unknown culture components will vary unpredictably with wavelength, but are perhaps not significant in the 800-870 nm region in comparison to the signal from the proteins of interest. I would try using the OD at the trough around 825 nm to correct both the LHC absorbance around 803 and the Bchla absorbance around 850-860 nm].

For absorbance spectra, cell material (either a colony suspended in ~ 10 µl of sterile buffer or wet cell pellet from 1.5 –15 ml culture) was sandwiched between 22 mm coverslips and placed against the front wall of a 6-position cuvette holder. A tiny drop of

water was used if necessary to adhere the coverslips flush against the side of the holder. Absorbance was scanned between 400 and 1000 nm at 600 nm/min; the resulting curve was smoothed using a 7-point window. The manual Trace function was used to determine the wavelength and absorbance of peaks not identified by the automated peak-identification algorithm.

# Results

## Direct Plating and Isolation

After 1-2 weeks of incubation, many students using Sippewisset soil cores obtained at least some apparent PNS colonies on their plates with a diversity of colony types represented. One student who obtained a particularly high number of colonies had dissected away other portions of the mat core to use only the region containing the purple layer. Only one student of 10 obtained apparent PNS colonies from School Street Marsh soil; the several colonies obtained all had the same appearance: small, flat, pale pink colonies.

Most samples plated onto the various AT media produced at least some colonies of putative PNS bacteria, identified by colors ranging from pink or light orange through red, purple or brown. The highest number of colonies, and the greatest diversity of colony types, was consistently found on plates derived from water samples; sediment samples from the same location generally produced fewer colonies and soil samples the fewest. This may be simply a function of the amount of starting material; the ability to concentrate organisms from a water sample by filtration allows a larger amount of the environment to be sampled.

Two sample sites that did not produce apparent PNS colonies are Quisset Pond and Vineyard Haven; the roadside pond on Martha's Vineyard produced only one. The failure to obtain putative PNS from Vineyard Haven is not surprising, since it is a sandy beach area with low levels of organic material. The failure to obtain more colonies from the organic-rich margins of two freshwater ponds is more surprising to me. The sampling location in Quisset Pond was unique in having a dense population of submerged aquatic vegetation, and the roadside pond on Martha's Vineyard was unique in having a moderate density of duckweed floating on the surface. The significance of these observations is not at all clear to me. The plates inoculated from Quisset Pond were also the only plates incubated initially in the glovebox instead of in GasPak jars; the humidity and composition of the atmosphere and intensity of illumination differed in these environments but again, it is not clear to me how that may have contributed to the absence of PNS colonies.

Many apparent PNS colonies on the initial plates were near to or even surrounded by nonpigmented growth, and virtually all colonies from the initial plates examined under the microscope contained more than a single morphotype. Earlier attempts to pick putative PNS colonies may have resulted in fewer contaminating heterotrophs on subsequent plates; on the other hand many tiny pigmented colonies may have been

missed with a shorter incubation time. Many pinprick colonies were picked under the dissecting scope and patched onto a small section of agar (with up to 50 patches on a single plate). After subsequent incubation about half the patches showed good growth and could be streaked for isolation.

After monopolizing an increasing number of GasPak jars with my initial and restreaked plates, I transferred all my plates to the glovebox. For the first few days in the glovebox, growth on the restreaked plates apparently continued, and a large amount of water was removed daily from the lids of the plates. Growth seemed to slow thereafter; lightly pigmented areas of confluent growth on many plates didn't continue to expand; nor did isolated colonies develop. Although condensation on the inner surface the glovebox suggested to me that the atmosphere was saturated with water vapor, I began to suspect that the plates were drying – a suspicion that was confirmed with the appearance of cracks around the periphery of the agar. I probably created this phenomenon by the intensity of illumination. I had been concerned that illumination would be inadequate for a plate in the middle of a stack that might be half a meter or so from a light source, so I set up a bank of 6 bulbs outside the glovebox. Although I couldn't sense an increased temperature in the vicinity of the plates by my gloved hand, I probably set up a convection current of warmer air which evaporated water from the plates and deposited it on the cooler surface of the glovebox. After transferring plates back to GasPak jars, development of the plates continued and I was able to pick apparently isolated colonies from many of the plates. As it turns out, however, none of the plates restreaked from my own brackish samples produced isolated colonies in time, so the most productive habitat for PNS bacteria on the basis of the initial plating was not represented among the organisms transferred to liquid culture.

Growth on plates after restreaking represented a diverse range of colors, however, I did not consider the colors to be reliable clues about identity for several reasons. Most obviously, apparently identical colony color and morphology often corresponded to distinctly different cell types under the microscope, e.g., dark red domed colonies formed from either small ovococcoid cells or long tapered rods. Furthermore, the color after restreaking often was not the same as the color of the colony when first isolated, and the same colony streaked onto two different media often produced different color growth (even when cell morphology was indistinguishable). The most obvious example of this phenomenon was darker maroon growth on PNS-succinate plates and lighter red growth on PNS-ethanol plates; this occurred in every instance when examined and with at least two different strains based on morphology (ovococcoid cells and tapered rods).

## Photoheterotrophic and Photoautotrophic Liquid Culture

I transferred growth from 17 colonies to photoheterotrophic (acetate containing) liquid medium, and incubated the tubes as described above. I realized that in some cases I took the risk of transferring contaminants, since the colonies were derived from plates that clearly also contained nonpigmented growth. However, time constraints prevented me from performing additional restreaking, and all colonies transferred were both isolated and appeared to contain a single morphotype under the microscope. In retrospect, it might have been better had I been more careful about the amount of time the plates had spent in an oxic environment. Prior to picking colonies to inoculate tubes, I had left many of the plates on my bench several hours on one or two different occasions as I examined them, before transferring them back to anoxic conditions for further incubation. Once I inoculated the tubes and left the plates on my bench for several days, heterotrophic growth appeared on most of the plates, often surrounding the 'isolated' colonies I had picked. Reducing the exposure to air prior to picking colonies may have reduced the risk of transferring contaminants.

The following 17 isolates were each transferred to duplicate tubes at 3 different salt concentrations, a total of 6 tubes per isolate.

| | **Fresh** | **Brackish** | **Marine** | |
|---|---|---|---|---|
| **Martha's Vineyard** | MVF-CD | | MVM-QD<br>MVM-QS<br>MVM-QW1<br>MVM-QW2 | 5 Martha's Vineyard |
| **Woods Hole** | SmSR1<br>SmSR2<br>SmSR3 | SpBC<br>SpOK<br>SpSKR<br>SpPL1<br>SpPL2<br>SpMG | WHM-GD<br>WHM-GS<br>WHM-GW | 12 Woods Hole |
| | 4 fresh | 6 brackish | 7 marine | |

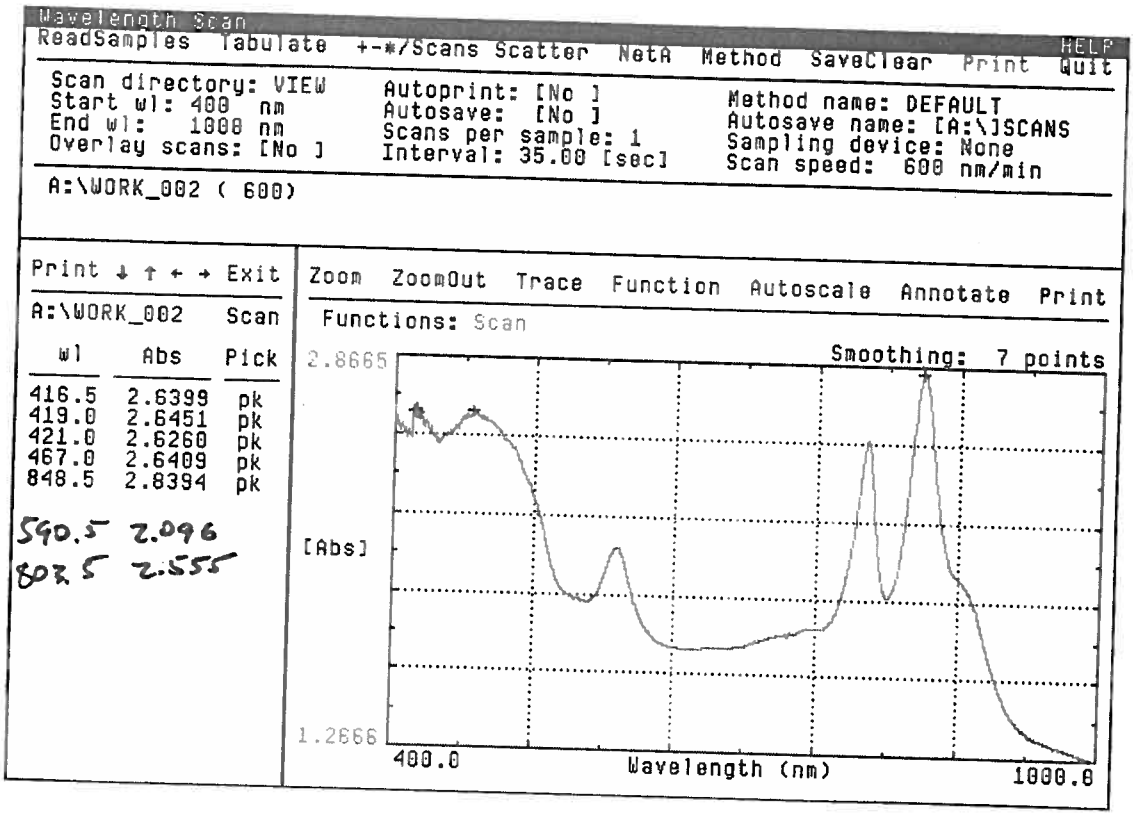
Isolates from Sippewisset and School Street are coded with the initials of the student who obtained them; other isolates are coded D, S, or W to indicate an origin from sediment, soil, or water, respectively. I chose these isolates because they were all the isolates I had that I characterized as rods; I was still hoping to find a number of closely related strains in order to do the biogeography project. Nonetheless, these strains represented a range from short tapered rods, essentially pear-shaped, to longer tapered rods, shaped like Pfennig bottles, to even more elongated forms, sometimes tapered on both ends to form spindles. Most colonies examined contained cells ranging over a subset of these morphologies, and I had sometimes noted changing cell morphologies from a single colony over time (generally progressing to shorter forms). Hence, I thought these isolates could conceivably represent pleiomorphic forms of a single species, which I would characterize as short to medium length rods, generally tapered on one end and occasionally on both, generally slightly curved especially when the taper is less

pronounced. I discriminated against isolates that were primarily coccoid or ovoid, or colonies with a greater diversity of morphotypes.

Growth was first detectable in a few of the tubes by eye after 3 days and in many of the tubes over the subsequent few days. Although spectrophotometer readings could have confirmed growth before this point, readings were suspect because an improvised tube holder in an old B&L Spectrophotonic 20 I was using didn't hold all tubes firmly enough to provide reliable readings at low optical density. Subsequent readings with a Genesys Spec20 and a proper tube holder were much more reliable. While the first tubes to show growth, MVF-CD in FAT-Ac medium, were distinctly pink while still only faintly turbid, none of the other isolates showed obvious color even at ODs over 0.1. I thought I may have only grown heterotrophic contaminants, so 5 days after inoculation I sacrificed the tube with the highest optical density, MVM-QW1 in MAT-Ac at an OD of 0.200 for spectral analysis. Pelleting the biomass from the entire 18 ml volume provided more than enough material for a spectrum showing clear peaks at 848.5 nm (bacteriochorophyll A), 803 nm (light harvesting complex) and 590.5 nm (carotenoids). Generally, yellow, orange, brown, or even a slightly green color did develop in the tubes over time, but only after cultures were fairly turbid. The only isolates that ever showed pink or red coloration in unopened tubes were the freshwater isolates, MVF-CD which was pink to bright red in liquid culture, and the three SmSR isolates which were faintly pink. However, a day or so after exposure to air, many of the yellow to brown tubes also assumed a pink or red tint to some degree, although never to the extent of MVF-CD.

Spectral analysis was eventually performed on all the isolates which grew in liquid media (except SmSR1 and SmSR2 which grew quite poorly and were considered likely to be identical to SmSR3). Although most strains were tested in different media and some were tested before and after the development of a pink color subsequent to air exposure, differences by more than 1 nm in the location of the two major peaks were never observed. Differences in the absorption maxima of the carotenoids are likely, but would have been difficult to observe with my spectra on intact cells, since in most spectra turbidity almost completely obscured this region.

| | LHC | | BCHLa | | |
|---|---|---|---|---|---|
| | 803 nm | 805 nm | 849 nm | 855 nm | 862 nm |
| MVF-CD | | X | | | X |
| Sm-SR3 | X | | | | X |
| MVM-QD<br>MVM-QW2<br>WHM-GS<br>SpBC | X | | | X | |
| MVM-QS<br>MVM-QW1<br>WHM-GW<br>SpOK | X | | X | | |




The first tubes to show growth for a particular isolate were always the tubes that matche the salinity of the source environment. This evidence supported my decision to treat the Sippewisset isolates as brackish; however, this may not truly reflect the environment, but only the fact that the PNS plates used for plating these samples had a salt content similar to my BAT media. The observation involves the only 2 Sippewisset isolates (of 6) that grew in any tube; one of these grew only in BAT while the other eventually grew in FAT also but not MAT. While the 6 of 7 marine isolates that grew seemed to come up in BAT medium almost as fast as they did in MAT, the freshwater isolates only showed growth in BAT much later than they did in FAT. If this is a general phenomenon, it may explain some of the difficulty we had in obtaining purple nonsulfur isolates from School Street Marsh on PNS plates containing an intermediate salt concentration. However, once again, the ability to draw inferences from this observation about freshwater isolates is tempered by a small sample size of only two organisms. I believe the three SmSR isolates all represent the same organism, which grew poorly at best in any of the liquid media.

Flocculent, clumped, or wall growth occurred in most of the tubes, sometimes developing only after turbidity was advanced, but often progressing along with dispersed growth in the liquid. In some cases, the liquid remained quite clear while clumped biomass developed extensively, and dispersed growth only occurred late e.g., more than a week after inoculation. While clumped growth was common in any case, it essentially always occurred when an isolate was grown in a salt environment unlike that from which it was isolated. Future work with these or similar organisms will have to devote consideration

to reliable estimates of growth rate, since clumping appears to be such an extensive phenomenon.

Eventually, 10 of the 17 isolates grew well enough in the initial photoheterotrophic tubes to be inoculated into both a second set of photoheterotroph tubes as well as photoautotrophic tubes. The inoculum in each case was the tube that had shown the best growth in the first set; e.g., a single tube of MVF-CD grown in FAT was used to inoculate FAT, BAT, and MAT media in the second set of photoheterotrophic tubes and in the photoautotrophic tubes. The second set of photoheterotrophic tubes in some respects confirmed the results from the first set: MVF-CD came up quickly in FAT, and more slowly in BAT. SmSR3 grew in FAT but only slowly, in clumps, and to a low density. The marine isolates came up fairly quickly in MAT. In two respects, however, the growth in the second set of photoheterotrophic tubes was troubling. First, the two Sippewisset isolates that had been transferred from BAT medium failed to grow in BAT on the second round, but did grow in MAT. In fact, nothing grew in the second set of BAT tubes. Perhaps the simplest explanation would be that I poisoned the medium somehow, but it was prepared as a common batch with the FAT and MAT media, and only separated for the addition of NaCl, filtration, and dispensing to tubes. If I had forgotten to add the salt to the batch of BAT, or added too much, I would have expected either MFV-CD or the marine isolates to respond appropriately, but they also failed to grow in this batch of BAT.

The second problem was evident with the marine isolates. All 6 did grow quickly and eventually obtained high OD in the second round of MAT, as expected, but all 6 also began quite early to show growth in FAT, which wasn't quite as fast as in MAT, but which did result in high cell density. One of the two Sippewisset isolates also showed fairly rapid growth in FAT only shortly after growth was evident in MAT. Most of these isolates had, in fact, shown growth in FAT during the first round as well, but only beginning quite late, progressing slowly, and attaining low density. Microscopic examination of the turbid 2$^{nd}$ round MAT cultures all showed a single cell type: short, tapered, pear shaped rods, arguably consistent with what I had seen in the colonies picked initially. Microscopic examination of turbid 2$^{nd}$ round FAT cultures showed one consistent morphotype distinct from that in MAT: longer, pointed rods, often with one or more phase-bright spherical inclusions. I had seen this morphology occasionally when examining growth from the direct plates, but never as the majority morphotype from a colony. I would conclude that this morphotype represents a freshwater-tolerant phototroph that was consistently present in the marine isolate and Sippewisset inocula for the second round of tubes. Since a single MAT tube (or BAT tube for the Sippewisset isolate) from the first round had been used to inoculate both the BAT and FAT media in the second round, this freshwater tolerant organism must also be capable of proliferating at higher salt concentrations. I don't have an explanation for why this organism didn't show earlier and more rapid growth during the first round, when it was presumably inoculated into the FAT tubes as well.

Growth in the photoautotrophic tubes, when it occurred, was fairly uniform in two respects: it always had a similar brownish tint, and it was always clumpy. Spectral

analysis of photoautotrophically grown biomass was not done. Jane Gibson wondered, since these tubes were only partially full and being shaken horizontally, if the clumpiness may have resulted from early wall growth that had been dislodged. However, there were no remnants of these growths on the walls, and I don't recall seeing any growth on the walls initially. Growth was first detected by faint turbidity, followed quickly by the formation of a few tiny clumps which grew larger and heavier. Accompanying the growth of the clumps was moderate development of turbidity, but without extensive formation of flocculent material I might have thought would be intermediate between the dispersed growth and clumped growth.

Only 2 of the 10 strains inoculated into photoautotrophic media failed to show any growth, although 3 of the 8 that grew only did so to low density. Strain MVF-CD, one of the most rapidly growing strains in photoheterotrophic growth, only developed turbidity fairly late under photoautotrophic conditions.

| Strain | Source | 1st Photoheterotrophic Tubes | | | 2nd Photoheterotrophic Tubes | | | Photoautotrophic Tubes | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | FAT | BAT | MAT | FAT | BAT | MAT | FAT | BAT | MAT |
| MVF-CD | fresh | + | late | late | ++ | - | + | late | late | - |
| SmSR1 | fresh | low | low | - | nd | nd | nd | nd | nd | nd |
| SmSR2 | fresh | low | low | - | nd | nd | nd | nd | nd | nd |
| SmSR3 | fresh | low | low | - | low | - | - | low | low | low |
| SpBC | brackish | - | + | - | - | - | + | - | - | - |
| SpOK | brackish | late | + | - | + | - | + | - | low | low |
| SpSKR | brackish | - | - | - | nd | nd | nd | nd | nd | nd |
| SpPL1 | brackish | - | - | - | nd | nd | nd | nd | nd | nd |
| SpPL2 | brackish | - | - | - | nd | nd | nd | nd | nd | nd |
| SpMG | brackish | - | - | - | nd | nd | nd | nd | nd | nd |
| MVM-QD | marine | late, low | + | + | + | - | ++ | late | + | + |
| MVM-QS | marine | late, low | + | + | late | - | ++ | - | - | - |
| MVM-QW1 | marine | late | + | + | + | - | ++ | low | low | + |
| MVM-QW2 | marine | late | + | + | late | - | ++ | low | + | + |
| WHM-GD | marine | - | + | + | nd | nd | nd | nd | nd | nd |
| WHM-GS | marine | late, low | + | late | + | - | ++ | low | - | low |
| WHM-GW | marine | late | + | - | + | - | ++ | + | + | + |

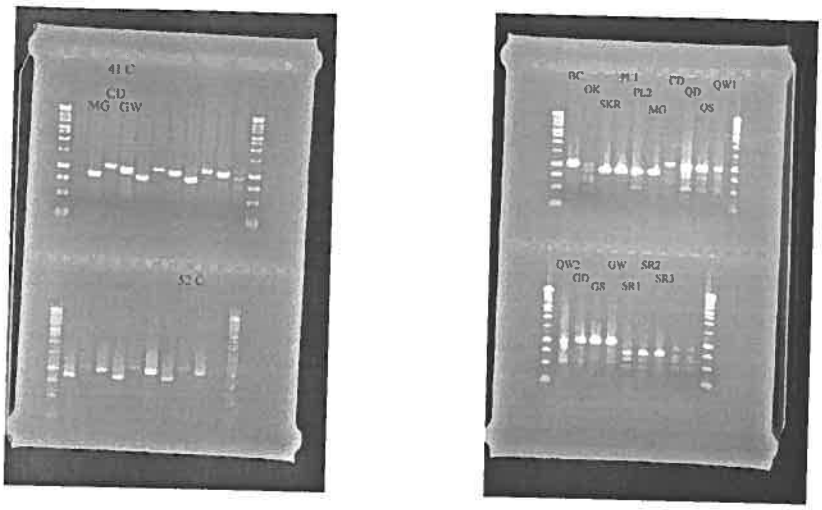
| | |
|---|---|
| ++ | rapid growth |
| + | growth |
| late | growth after long lag |
| low | growth to low density |
| - | no growth |
| nd | not determined |

## ITS and 16S Analysis

Shortly after the first set of photoheterotrophic growth tubes were inoculated, I needed to have PCR amplicons ready to be submitted for sequencing at an external facility. My original plan was to use the 16S rDNA sequence to identify isolates to the species level, and after choosing isolates of the same species to work with, using the ITS sequence to establish a phylogeny within the strain. As it turns out, I would need to have all the physiological work well underway before the sequencing results would be available. Hence, I had to decide on other grounds which organisms to work with, and perform sequence analysis after the fact.

Because the ITS work involved a new set of primers, my first step with the attempt to amplify the ITS region was to empirically determine an optimum annealing temperature – i.e., the lowest annealing temperature that produced a single distinct band. The ITS primers are relatively short (16- and 17-mers) and AT rich, so the predicted melting temperatures were 41 C and 43 C. These temperatures are fairly low for PCR primers; I was concerned about possible non-specific amplification. For the first test, I chose 3 organisms originating from different environments and representing the range of cell morphotypes I had chosen to work with: SpMG, MVF-CD, and WHM-GW. 6 reactions were set up for each organism, and amplified in the gradient thermocycler at the following annealing temperatures: 41 C, 42.7 C, 43.9 C, 45.4 C, 48.4 C, and 51.9 C. All the amplifications were successful, with a single bright band visible at 41 C for all 3 organisms, and decreasing band intensity for each organism as temperature increased. Furthermore the band size was different for each organism, suggesting that the length of the ITS amplicons alone would be useful in grouping the organisms.

I then attempted to amplify the ITS region from the remaining 14 organisms using a 41 C annealing temperature. Most of the reactions produced a single bright band; however, several organisms produced additional, fainter bands or extensive smearing as well. One organism, SpOK, didn't produce any single bright band, only a number of fainter ones. The negative controls in this reaction also produced faint multiple bands, indicating some level of contaminating DNA in the reactions. Nonetheless, I now had ITS size information from 16 of the isolates, and decided to submit 14 of these amplicons for sequencing. The two I chose not to submit were SmSR1 and 2, which I believed were the same organism as SmSR3. The SmSR3 ITS region was chosen for sequencing because the PCR reaction didn't produce fainter contaminating bands. PCR products were purified with the Promega Wizard PCR Cleanup Kit prior to quantification by absorbance at 260 and 280 nm, and submitted for sequencing with the 1491 F primer.




ITS amplification gels: Initial annealing temperature gradient PCR reaction products (left), and subsequent 41 C annealing temperature reaction product (right). Molecular size standards in all outside lanes have the 5 smallest bands at 250, 500, 750, 1000, and 1500 kb.

The initial attempt to amplify the 16S gene using standard bacterial 8F and 1492R primers from all isolates produced faint bands of the appropriate size from all isolates, but the bands were vanishingly faint from 3 isolates, MVM-QS, MVM-QW2, and SmSR1. The presence of intact colony material from these isolates in the NP-40 lysate tubes used to prepare template DNA led me to believe that low template concentration may have been the main problem in these three reactions. I pipetted the colony material and remaining original cell lysate up and down several times in the smallest pipette tips, repeated the freeze-thaw cycles, and repeated the pipetting operation. Two additional PCR reactions were prepared from each isolate with either 1 or 5 ml of the new cell lysate as template material. Both reactions for MVM-QS and MVM-QW2 produced single bright bands at the expected positions, but neither reaction for SmSR1 was successful.

By this point I had obtained the ITS size information from most of the isolates. Particularly since the ITS sizes seemed to group organisms that I already considered similar based on cell morphology, source, and limited spectral absorbance data, I decided that I would only sequence one or two organisms from each group of what I thought likely to be related organisms. Organisms chosen for 16S sequencing were MVM-CD, SmSR3, SpBC, SpSKR, SpMG, MVM-QS, and WHM-GD.

Since the initial PCR amplification of the 16S gene had produced very little product, I performed additional duplicate 100 µl reactions for each of these 7 organisms. All reactions were successful, reactions from the same organism were pooled for purification using the Quiagen Quiaquick PCR cleanup kit, quantified, and submitted for sequencing using the 8F primer.

## Phylogenetic Analysis

The chromatograms of the 16S sequences returned from the sequencing facility were examined using the Chromas software available as freeware on the internet. 6 of the 7 sequences looked quite good, with clean, uniformly spaced, non-overlapping peaks clearly distinguished from the background over much of the sequence, and only the expected deterioration in signal quality with an increasing frequency of ambiguous nucleotides beyond about 650 nucleotides. However, the sequence from SmSR3 had numerous ambiguous nucleotides in the first several hundred nucleotides as well. Examination of the chromatogram showed not a garbled absence of signal, but clear signals apparently derived from two different 16S sequences. In many places, distinct smaller peaks were nested beneath the larger peaks, but the sequencing software called the base to represent the larger peak. In places where the peaks were of approximately equal height, the base call was ambiguous. There were also numerous regions where the dual signal disappeared, presumably regions where the two sequences were identical and in register. The dual signal appeared to end after several hundred nucleotides, however; the remainder of the sequence seemed to be of good quality until the usual deterioration of signal quality at the end. The origin of the two sequences produced in the PCR reaction is unclear, as is the reason why the apparent differences appeared only in the 5' region of the molecule. One possibility is the presence of two or more 16S rDNA genes in isolate SmSR3 which differ considerably from each other, but only in the early portion of the sequence. Another possibility is a chimeric sequence formed from a partial length amplification of contaminating DNA and partial amplification of the intended template. If such a chimera formed in an early PCR reaction cycle, it may have continued to be amplified to generate a significant minority of the full length products. I generated two versions of this sequence for further analysis: a longer sequence that included the early, ambiguous nucleotides and a shorter sequence which omitted them.

Sequences were imported to Arb, aligned with the Fast Aligner algorithm to the 3 nearest neighbors in the database, and then inspected and further aligned manually by comparison to sequences from purple nonsulfur bacteria from each of the major groups of PNS bacteria in the alpha and beta Proteobacteria. The presence of several unambiguously aligned helical stem structures, which were longer in the beta Proteobacterial sequences (*Rubrivivax*) and SmSR3 in comparison to the alpha Proteobacterial sequences, strongly suggested that this isolate was within or related to the beta Proteobacteria. I decided to terminate all sequences after helix 22 (according to Arb helix numbering) in order to avoid including sequence of dubious quality. I expected approximately 600 nucleotides including several variable regions in the first third of the molecule would be adequate to place these sequences in a phylogenetic tree. Once all sequences were satisfactorily aligned, I constructed a neighbor joining tree using for reference sequences not only representatives of all the known groups of PNS bacteria, but also all major lineages within the alpha and beta Proteobacteria. I also included one sequence each from purple sulfur bacteria (*Chromatia*), a *Pseudomonas* group, the Vibrionaceae, and the enteric bacteria in the gamma Proteobacteria. All 7 sequences from my isolates clustered closely with known purple nonsulfur bacteria. Both the short and long version of sequence from SmSR3 grouped with *Rubrivivax gelatinosus* in the

beta Proteobacteria, as I had already surmised. The pink-red freshwater isolate MVF-CD grouped with *Rhodopseudomonas palustris*, consistent with Jane Gibson's and Carrie Harwood's tentative identification of it based on cell morphology and growth patterns. The remaining isolates, all marine in origin, fell within a clade including the *Rhodobacter* genus, consistent with the pear shaped cell morphologies that were the dominant form after growth in MAT photoheterotrophic tubes. However, the isolates were not specifically related to the *Rhodobacters* – instead, they clustered, not surprisingly, with marine PNS bacteria. Isolate SpMG clustered with *Rhodovulum euryhalinum*, and SpBC, SpSKR, MVM-QS, and WH-MD with *Rhodovulum sulfidophilum*.