Isolation of Chemoautotrophic Sulfur-Oxidizing Denitrifying Bacteria

from a Stratified Marine Ecosystem: Salt Pond

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Microbial community structure within a meromictic lake ecosystem

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Abstract

Meromictic lakes are stratified ecosystems characterized by clines of oxygen, salinity and temperature with concomitant changes in redox chemistry spanning the vertical depth continuum. In order to better understand the microbial community structure associated with low oxygen metabolism within these ecosystems, a cultivation-independent survey targeting the bacterial small subunit ribosomal RNA V6 region coupled to physical and chemical analyses was conducted in Salt Pond on the coast of Falmouth Massachusetts. Analysis of V6-tag pyrosequence reads derived from six depths spanning the oxycline identified Actinobacteria, Bacteroidetes, Cyanobacteria, alpha and gamma Proteobacteria as abundant community members within the oxic mixolimnion. Within the oxycline, cyanobacteria gave way to green sulfur and purple sulfur bacteria. Within the monimolimnion green sulfur bacteria represented over 60% of all V6-sequence tag data followed by firmicutes, alpha, delta, epsilon and gamma Proteobacteria. Survey data was complimented by efforts to isolate sulfur-oxidizing microbes with the capacity to use nitrate as the sole terminal electron acceptor. Sulfide concentrations ranging between 0.4-1.2 mM were measured within the anoxic monimolimnion. Efforts to cultivate sulfur-oxidizing organisms below the oxycline yielded three strains of Thiomicrospira closely related to Thioalkalimicrobium aerophilum nested within the gamma Proteobacteria and two divergent groups of epsilon Proteobacteria related to Sulfurimonas autotrophica and Arcobacter nitrofigilis respectively. Overall, microbial community structure in Salt Pond was dominated by phototrophic organisms spanning the vertical depth continuum with chemotrophic energy metabolism based in part on the conversion of reduced sulfur compounds increasing with depth below the oxycline.
Introduction

Meromictic lake ecosystems create stratified niche space for a wide-array of photosynthetic and chemosynthetic microorganisms. Broadly defined, they can be separated into an upper oxic mixolimnion and a lower anoxic monimolimnion. Between these two regions is a sharp and relatively stable redox transition zone of decreasing oxygen tension called the oxycline or chemocline hosting an array of photosynthetic and chemosynthetic microorganisms [1-8]. Meromictic lakes are useful systems to investigate microbial community structure along defined redox gradients based on the residual chemical energy of ammonia, methane, transition metals including iron and manganese, hydrogen sulfide and molecular hydrogen [9-16], and resemble the stratified chemical gradients typically associated with anoxic marine sediments [17]. Therefore, biogeochemical and microbial ecological studies of meromictic lakes should be extensible to stratified aquatic ecosystems including marine oxygen minimum zones (OMZ) associated with enclosed basins and coastal fjords.

OMZs are a recurring oceanographic feature. They provide a physical and chemical boundary layer for many organisms, expose large sections of the seafloor to long-term anoxic conditions and support the biogeochemical transformation of reduced nitrogen compounds and methane to nitrous oxide and carbon dioxide respectively. Recent cultivation-independent molecular surveys of OMZ systems in Namibia [18], Peru [19] and the subarctic Pacific Ocean (unpublished observations) have recovered an abundance of small subunit ribosomal DNA (SSU rDNA) sequences closely affiliated with chemosynthetic sulfur-oxidizing bacteria including free-living relatives of hydrothermal vent clam and mussel endosymbionts. The widespread occurrence of putative sulfur-oxidizing bacteria within marine OMZs raises fundamental
questions about the mechanism and biogeochemical role of sulfur cycling within the interior ocean.

Salt Pond is a shallow seasonally stratified meromictic lake situated on the Massachusetts coast near the town of Falmouth. Based on previous studies, the water column chemistry of Salt Pond resembles that of other stratified marine ecosystems [20, 21]. Ease of access combined with representative water column chemistry make Salt Pond an ideal location for exploring microbial community structure along defined physical and chemical gradients. Previous studies of the Salt Pond microbiota focused on enrichment of magnetotactic bacteria with special emphasis on morphotypic variation and contribution to chemical cycling [20-22]. Although non-magnetotactic bacteria were identified as background in these studies, to date there have been no efforts to explore the overall microbial community structure of the Salt Pond ecosystem.

Here we report the results of high resolution studies aimed at profiling the Salt Pond microbiota along defined gradients of oxygen, temperature, light intensity, salinity, and sulfide using a combination of V6-tag pyrosequencing and cultivation techniques using sulfide or thiosulfate as electron donors and nitrate as sole electron acceptor to better understand biogeochemical controls on microbial community structure and metabolic potential. The data reflects summer stratification conditions during field collection in July 2008.

Methods

Sampling depths began at the base of the mixolimnion (1.82 m), and continued through the oxycline (2.2 m, 2.9 m, 3.1 m), down to the lower monimolimnion (3.4 m, and 3.7 m). Water samples were collected using a peristaltic pump (Cole-Parmer) system powered by a marine battery pack and DC power converter with ¼” tubing directly tethered to a CTD array. The array
was manually deployed from a small rowboat in the northwestern quadrant of Salt Pond. Temperature, Salinity, depth, light intensity and oxygen were recorded at approximately 0.25 m intervals down to base of the monimolimnion (4.04 m). Water samples were pumped directly into autoclaved 1 L bottles for environmental DNA (eDNA) extraction and cultivation work. Aliquots from each bottle were transferred into 15 ml conical tubes amended with either 1% formaldehyde (final concentration) for cell counts or zinc acetate for downstream sulfide measurements, according to the methods of Cline [23].

**Sulfide Analysis**

900µl from each zinc acetate treated sample was transferred into 2.0 ml disposable cuvettes and amended with solutions 1 and 2 using a hydrogen sulfide detection kit (HS-WR) according to the manufacturer’s instructions (Hach). Samples were subsequently analyzed on a Carey 50-scan UV-visible spectrophotometer at a wavelength of 670 nanometers. 1:100 dilutions for samples below the transition zone (3.4 m, and 3.7 m ) were also prepared and measured in order to remain with the dynamic range of the spectrophotometer. A standard curve was generated based on serial dilutions of a 100mM Na₂S stock solution.

**Environmental DNA Extraction**

100 ml from each depth interval was filtered onto 47mm 0.2µM GTTP membranes (Millipore). Membranes were subsequently sectioned and transferred into 2.0ml PowerBead tubes (MoBio) and homogenized for 30 seconds using a bead beater device (Biospec Products). eDNA was extracted according to manufacturer’s instructions, eluted in 100µl of 10mM Tris and stored at -20°C until needed for downstream PCR experiments. eDNA quality was determined by running
5μL samples and 100ng of 1KB+ Labber (New England Biolabs) on 1% agarose gels in 0.5x TBE for 10 hours at 50 volts.

Environmental DNA Amplification and Colony PCR

For quality control purposes prior to V6-tag sequencing, small subunit (SSU) ribosomal DNA (rDNA) bacterial sequences were amplified via PCR using B27F (5’AGAGTTTGATCCTGGCTCAG) and the universal primer 1492R primer (5’GGTTACCTTAGTTACGACTT) using the following PCR profile: a 2 min hot start at 95°C followed by 30 cycles of denaturation (20 sec at 95°C), annealing (20 sec at 55°C), and extension (1 min at 72°C) and a final extension at 72°C for 3 minutes. Each 25μL reaction contained 1μL of template DNA, in 1X master mix (Promega) supplemented with 1.25 μL of reverse and forward primer (10 pmol each). The same primer pair and amplification procedure was used in colony PCR experiments to identify Salt Pond isolates (see below) following 10 minute denaturation in 0.05% NP40 solution at 95°C.

PCR Amplicon Library Construction for Pyrosequencing

Primers targeting the V6 hypervariable region of bacterial 16S rRNAs (Escherichia coli positions 967-1046) were used to generate amplicons for pyrosequencing [24]. The oligonucleotide design included 454 Life Science's A or B sequencing adapter (shown in lowercase in the following) fused to the 5’ end of primer A-967F, 5’-gcctctggctgccgcatcag-CAACGCGAAGAACCTTACC-3’ , and B-1046R, 5’-gccttgccagccgctcag-CGACAGCCATGCANCACCT-3’. PCR amplicon libraries for individual depths included linker barcodes enabling multiplexed sequencing.
reactions [25]. Amplification, quality control and data analysis procedures followed previously described methods for V6-tag sequences [24].

*Enrichment, Isolation and Culture Conditions*

To enrich for sulfur-oxidizing denitrifiers, a modular medium buffered with 10mM MOPS pH 7.2 containing 1X seawater base amended with salts (10mM NH₄Cl, 1.5 mM KH₂PO₄), cofactors (12X Vitamins, B₁₂, and trace elements) was used. The carbon source supplied to the medium was 2mM Na₂HCO₃. Electron donors included 8mM Na₂S or 30 mM NaS₂O₃ with 20mM NaNO₃ as sole electron acceptor.

Primary enrichment cultures were generated at 26°C or 30°C using a modified version of the agarose-plug overlay method developed by Holger Jannasch (1% GTG agarose in plug containing sulfide and bicarbonate, and 0.16% in overlay containing modular medium mixed with Salt Pond inocculum) to isolate individual colony forming units along oxygen-sulfide gradient. Primary isolates obtained from enrichments were subsequently transferred to 500 µl of 1X seawater base and used to inoculate fresh overlays or 3% GTG agarose plates containing modified overlay media (30 mM thiosulfate substituted for sulfide as electron donor with 40 mM Na₂HCO₃ as carbon source and pH buffer under anaerobic growth conditions).

*Phylogenetic Analysis*

Individual colonies from selected plates were selected for colony PCR as described above. Sequence traces were obtained using BigDye chemistry on an ABI Prism 3730 sequencer, trimmed using a Unix implementation of Phred [26] (phred -id chromat_dir -pd phd_dir -sd edit_dir -trim_alt unused -trim_phd -trim_fasta -trim_cutoff .01 -qd qual_dir) and analyzed using the
ARB package for phylogenetic sequence analysis [27]. Sequences were imported into the full-length SILVA 92 database (www.arb-silva.de) [28] and aligned to the closest relative. For phylogenetic analysis, Salt Pond and selected reference sequences were exported from ARB and realigned using the multi-sequence alignment tool MUSCLE [29]. The resulting alignment was imported into MacClade [30] for manual refinement. Phylogenetic trees were inferred by PHYML [31] using an HKY + $\Gamma + I$ model of nucleotide evolution where the $\alpha$ parameter of the $\Gamma$ distribution, the proportion of invariable sites, and the transition/transversion ratio were estimated for each dataset. The confidence of each node was determined by assembling a consensus tree of 100 bootstrap replicates.

**Results**

**Objectives and Workflow**

The primary objectives of this study were to (i) profile the microbial community structure of Salt Pond as a function of oxygen, salinity and sulfide gradients using V6-tag pyrosequencing approaches, and (ii) to develop and ground truth methods for the isolation of chemoautotrophic sulfur-oxidizing denitrifying bacteria extensible to stratified aquatic ecosystems in general. Figure 1 provides a general workflow for the study. Following site selection and chemical profiling, both cultivation-independent and cultivation-dependent methods were employed. Both methods intersected at the use of SSU rDNA sequence information to infer phylogenetic relationships among taxa at both the community and isolate levels.
Water Chemistry and Sample Selection

Figure 2 describes water column chemistry collected on the afternoon of July 5th 2008. Density profiles through the water column reveal a dramatic shift in salinity between 1.0 and 1.5 m with a corresponding thermocline ranging between 26.25 °C at the surface to 16.01 °C at 4.03 m corresponding to the base of the monimolimnion (Figure 3). Salt Pond waters at the time of sampling were brackish peaking at 25.38 ppt salinity at the base of the monimolimnion. The oxycline covered a 1.6 m interval between 1.5 and 3.1 m. Sulfide concentrations increased sharply below 3.1 m, reaching 1.26 mM at the base of the monimolimnion. Water samples for eDNA extraction and cultivation work we selected on the basis of water column chemistry to bracket the oxycline and monimolimnion (see methods).

Microbial Community Diversity and Structure

To evaluate overall microbial community diversity and structure high resolution V6-tag pyrosequencing was conducted on eDNA derived from six depths spanning the oxycline and monimolimnion (see methods). A total of 210,632 tag sequence reads were generated according to the following distribution (1.8 m = 45,633, 2.2 m = 38,945, 2.9 m = 41,523, 3.1 m = 26717, 3.4 m = 29840 and 3.7 m = 27974 reads). Community diversity was evaluated using rarefraction analysis while community structure was evaluated at the phylum and order levels to provide both course and fine scale resolution within the accuracy bounds of variable-tag identification schemes (see methods). Overall, rarefraction curves for each of the six depth intervals exhibited similar slopes at the 3% identity cut-off (Figure 4). At this level of genetic distance, the curves
predict that additional sampling will lead to only modest increases in estimates of total diversity consistent with near saturation coverage of the V6 region for the six depth intervals.

Depth specific trends in the distribution of taxa spanning the vertical depth continuum were evident (Figures 5). At the phylum level (Figure 5A), Actinobacteria comprised between 22-27% of tag sequences in the first two depth intervals before falling to 3% within the monimolimnion. Cyanobacteria, Bacteroidetes, Verrucomicrobia, alpha and gamma Proteobacteria accounted for the remainder of tags within both intervals. Within the oxycline at 2.9 m, cyanobacteria gave way to green sulfur (Chlorobi) and purple sulfur bacteria (Chromatiales). Within the monimolimnion green sulfur bacteria represented over 60% of all V6-sequence tag data followed by firmicutes, alpha, delta, epsilon and gamma Proteobacteria. A closer look within the Proteobacteria revealed depth specific increases of V6-tags affiliated with known sulfate-reducing bacteria within the order Desulfobacterales reaching 5.47% of total tags at the base of the monimolimnion (Figure 5B). V6-tags affiliated with members of the Desulfuromanales were also identified below the 2.2 m depth interval. However, Desulfuromanales comprised less than 1% of tag sequences to the base of the monimolimnion. In addition to sulfate reducing bacteria, V6-tags affiliated with known sulfur-oxidizing bacteria including Arcobacter (Camylobacterales) and Thiomicrospira (Thiotrichaes) were also detected at low levels throughout the depth continuum.

Cultivation of sulfur-oxidizing bacteria

Figure 6 provides a general workflow for the cultivation approach developed in this study. Primary enrichments in agar-plug overlay tubes containing a sulfide gradient (revealed by the
color phase of the redox indicator resazurin were used as seed stock for secondary enrichment and isolation on thiosulfate containing plates (see methods). Although many isolates were obtained from enrichments spanning the depth continuum, only five were selected from downstream characterization based on their capacity to form colonies on agarose plates. In general, colonies formed in regions of the overlay that seemed to recapitulate aspects of their in situ environment. For instance, tubes inoculated with water from the 3.7 m depth interval formed colonies closer to the agar plug, whereas tubes inoculated with water from the 1.8 m depth interval formed colonies closer further away from the agar plug. In general, colonies formed within the agar-plug overlay medium over the course 3-5 days. On plates incubated in anaerobic jars colony forming units grew more slowly over the course of 7-10 days. In all cases but one, colonies appeared white when viewed under direct lighting presumably due to the precipitation of elemental sulfur granules. Photomicrographs appear in the inverse due to bottom illumination through the dissecting microscope (Figure 7). Although nitrate was used as sole electron acceptor in both agar-plug overlay and plate growth media, it remains formally possible that strains isolated in this study are capable of utilizing molecular oxygen as a terminal electron acceptor. Physiological characterization of the five isolates remains the object of ongoing investigation. However, phylogenetic classification based on SSU rDNA sequence (see methods) placed all five within known sulfur-oxidizing lineages (Figure 8). Three of the five isolates were affiliated with Thiomicrospira within the gamma proteobacteria. The remaining two were most closely related to environmental clones affiliated with Sulfurimonas and Arcobacter respectively within the epsilon Proteobacteria. These results are consistent with V6-tag sequencing data indicating the presence of all three groups within the Salt Pond depth continuum. Based on this observation,
it appears that the methods developed in this study for the isolation of sulfur-oxidizing bacteria are robust.

Conclusions

The current study provides a high-resolution look at microbial community diversity spanning defined gradients of salinity, oxygen and sulfide in the meromictic Salt Pond. Overall, microbial community structure is dominated by the phototrophic bacteria, with oxygenic photosynthesis giving way to low-light adapted anoxygenic photosynthesis within the oxycline and monimolimnion. It will be of interest to determine the taxonomic identity of green sulfur and purple sulfur bacteria resident with the depth continuum and to relate their V6-tag abundance with lipid biomarker or pigment analysis. Likewise a more thorough evaluation of V6-tag sequences in relation to published studies of magnetotactic bacterial enrichments from Salt Pond is warranted.

Based on V6-tag sequence analysis the Salt Pond site harbors a low level representation of indigenous chemoautotrophic sulfur oxidizing bacteria. Through the combined use of agar-plug and plate isolation techniques 5 isolates were obtained from within these lineages (three Thiomicrospira all close relatives of a described lineage, Thioalkalimicrobium aerophilum [32], one Sulfurimonas, and one Arcobacter sp). The later two isolates are novel with ~8% sequence divergence from known representatives. Taken together, the techniques employed in this study appear robust, capable of selecting specific physiological groups from within a diverse community background and should be tunable to other stratified aquatic ecosystems. Future studies focused on determining the precise metabolic capabilities of sulfur oxidizing isolates
obtained from Salt Pond including oxygen tolerance, growth rates and range of electron acceptors is necessary. Given their capacity to grow as colony forming units on agarose plates the potential for genetic analysis exists should they prove metabolically interesting. Future studies of Salt Pond focusing on the biogeochemical roles of phototrophic, sulfur-oxidizing and sulfate reducing community members have the potential to shed light on sulfur cycling within other stratified aquatic ecosystems including coastal waters and enclosed basins.

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Figure Legends

**Figure 1.** A workflow summary of Salth Pond sampling and analysis based on a preliminary chemical and physical profile followed by a combination of cultivation-independent molecular surveys and targeted isolation methods associated with specific metabolic subsystems, elemental cycles, or redox chemistries.

**Figure 2.** Opposing gradients of Salinity, Oxygen and Sulfide. Sampling depths for eDNA extraction and cultivation work are indicated with arrows.

**Figure 3.** Changes in light intensity and temperature as a function of depth.

**Figure 4.** Rarefaction analysis for V6-tag sequences derived from six sampling intervals spanning the Salt Pond depth continuum based on pairwise distance. Rarefaction is shown for OTUs with differences that do not exceed 3%.

**Figure 5.** (A) Phylum level classification of V6-tag sequences based on best blast hit analysis using the V6RefDB database (see methods) (B) Order level classification of V6-tags within the Proteobacteria. Values on the X-axis are shown as a percentage of total tags for the corresponding depth interval. NA represents unassigned tags within a given classification level.

**Figure 6.** A workflow summary of Salth Pond methods used to enrich and isolate sulfur-oxididizing bacteria from the Salt Pond depth continuum. P₀ represents primary enrichment
followed by successive selection and re-isolation using the agar-plug overlay method. Individual colonies derived from the primary enrichment were diluted in 1X seawater base and used to simultaneously inoculate a second sulfide gradient tube and GTG agarose plate containing thiosulfate as sole electron acceptor (see methods). Colony PCR was performed on selected colonies to ascertain the taxonomic identity of isolates (see methods).

**Figure 7.** Colony morphology of selected Salt Pond isolates grown on thiosulfate. SP_A4 corresponds to Salt Pond cast A4. Isolate designations use a standard nomenclature where the first number indicates depth interval: 1 = 1.82 m, 2 = 2.2 m 3= 2.9 m, 4 = 3.1 m, 5 = 3.4 m, and 6 = 3.7 m. The second letter indicates growth temperature (A = 26°C and B = 30°C). The third number indicates isolate id followed by the number of plate passages.

**Figure 8.** Distance tree of Proteobacterial SSU rDNA sequences isolated from the Salt Pond depth continuum. Bootstrap values (%) are based on 100 replicates using the maximum likelihood method and are shown for branches with greater than 50% support (see methods). The scale bar represents 0.05 substitutions per site. Isolate depths are color coded in the tree.
References

Salt Pond SP_A4 Opposing Gradients of Salinity, Oxygen and Sulfide

Figure 2
Salt Pond SP_A4 Light Intensity vs Temperature

Light Intensity (PAR)

Depth (m)

Temperature (°C)

Figure 3
Figure 4

Operational Taxonomic Units

No. of V6-tags Sampled

0.03 sequence identity cut-off

1.8m
2.2m
2.9m
3.1m
3.4m
3.7m
Figure 6

Sample Site

Salt Pond

Primary Enrichment

P₀

Secondary Enrichment

Agar-Plug HS-Gradient

Plate Isolation

Thiosulfate Anaerobic Jars

P₁