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Coupled reductive and oxidative sulfur cycling in the phototrophic plate of a meromictic lake

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ABSTRACT

Mahoney Lake represents an extreme meromictic model system and is a valuable site for examining the organisms and processes that sustain photic zone euxinia (PZE). A single population of purple sulfur bacteria (PSB) living in a dense phototrophic plate in the chemocline is responsible for most of the primary production in Mahoney Lake. Here, we present metagenomic data from this phototrophic plate – including the genome of the major PSB, as obtained from both a highly enriched culture and from the metagenomic data – as well as evidence for multiple other taxa that contribute to the oxidative sulfur cycle and to sulfate reduction. The planktonic PSB is a member of the *Chromatiaceae*, here renamed *Thiohalocapsa* sp. strain ML1. It produces the carotenoid okenone, yet its closest relatives are benthic PSB isolates, a finding that may complicate the use of okenone (okenane) as a biomarker for ancient PZE. Favorable thermodynamics for non-phototrophic sulfide oxidation and sulfate reduction reactions also occur in the plate, and a suite of organisms capable of oxidizing and reducing sulfur is apparent in the metagenome. Fluctuating supplies of both reduced carbon and reduced sulfur to the chemocline may partly account for the diversity of both autotrophic and heterotrophic species. Collectively, the data demonstrate the physiological potential for maintaining complex sulfur and carbon cycles in an anoxic water column, driven by the input of exogenous organic matter. This is consistent with suggestions that high levels of oxygenic primary production maintain episodes of PZE in Earth's history and that such communities should support a diversity of sulfur cycle reactions.

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INTRODUCTION

Euxinic conditions have been an intermittent feature of global oceans throughout Earth's history. They accompanied significant events such as Phanerozoic biotic crises (e.g., Pancost *et al.*, 2004; Grice *et al.*, 2005) and perhaps were common in the Mesoproterozoic (Canfield, 1998; Brocks *et al.*, 2005; Reinhard *et al.*, 2013). Although rare today, persistently euxinic environments serve as modern analogues to ancient water columns and their associated sedimentary basins. Of these modern analogues, meromictic lakes have been of intense interest due to their

phototrophic chemocline communities, large geochemical gradients, and laminated sedimentary records that can preserve biosignatures (Meyer & Kump, 2008). Mahoney Lake (ML) is a small meromictic lake in British Columbia, Canada, that represents an extreme end-member of such euxinia. The hypolimnion of the lake contains high levels of sulfide (30–35 mM). ML has a pH in the range 7–9 and thus is not a soda lake (Northcote & Hall, 1983). Its dominant anion is sulfate (400–500 mM), which is supplied by local surface waters draining alkaline lavas rich in Mg^{2+}, Ca^{2+}, Na^{+}, and $SO_4{}^{2-}$, and $CO_3{}^{2-}$ (Northcote & Hall, 1983). The hypolimnion is anoxic, eutrophic, and very saline (~74 g L^{-1} total dissolved solids (TDS)), while the epilimnion is oxic, oligotrophic, and of lower salinity (~28 g L^{-1} TDS) (Northcote & Hall, 1983).

Mahoney Lake (ML) is stable year-round due to density stratification, and a 10- to 20-cm-thick chemocline occurs at a depth of about 7 m. This layer supports a dense (~10^8 cells mL^{-1}) layer of anoxygenic phototrophs, limiting the mixing of nutrients to the epilimnion and absorbing all visible light (Overmann *et al.*, 1991, 1996a, 1996b). Primary productivity has largely been attributed to a single purple sulfur bacterium (PSB), which was originally classified as a *Thiocapsa* sp. based on cell morphology (Northcote & Halsey, 1969). However, cultivation studies yielded an okenone-producing member of the family *Chromatiaceae*, denoted strain ML1. Okenone, a biomarker for some species of PSB (e.g., Brocks & Schaeffer, 2008), is abundant in the lake sediments (Overmann *et al.*, 1993; Coolen & Overmann, 1998). Based on morphological and physiological properties, strain ML1 was reclassified as *Amoebobacter purpureus* (Overmann *et al.*, 1991) and was subsequently reclassified again to the genus *Lamprocystis* (Imhoff, 2001). More recently, 16S rRNA and *pufLM* gene sequencing revealed that strain ML1 is only distantly related to the type strain of *Lamprocystis purpurea* (Coolen & Overmann, 1998; Tank *et al.*, 2009).

While small numbers of green sulfur bacteria (GSB) are also present in and below the chemocline, the total sulfide oxidation rate cannot be attributed to PSB and GSB alone (Overmann *et al.*, 1996a). Chemoautotrophic sulfide oxidation has been observed near the oxic–anoxic interfaces of other stratified systems and by analogy may be expected in Mahoney Lake (Hadas *et al.*, 2001; Bühring *et al.*, 2011). Analysis of 16S rRNA gene sequences supports this, showing the presence of Epsilonproteobacteria that are likely to be sulfide and/or sulfur oxidizers (Klepac-Ceraj *et al.*, 2012). Sulfate reduction also occurs in the chemocline, and *in situ* generation is thought to supply much of the sulfide necessary to support anoxygenic photosynthesis (Overmann *et al.*, 1991, 1996a).

Collectively, these data indicate that complex cycling of carbon and sulfur occurs in the Mahoney Lake chemocline. The ML chemocline maintains a stable ecosystem in which sulfate-reducing bacteria supply sulfide for both phototrophic and chemolithoautotrophic primary productivity. These primary producers in turn supply some of the organic carbon necessary to support the heterotrophic sulfate reducers, resulting in a coupled reductive and oxidative sulfur cycle. Seasonal analyses of primary productivity and sulfur oxidation and reduction rates (Overmann *et al.*, 1996a), and modeling of the carbon cycle in the chemocline (Overmann, 1997), indicate that the system must depend on additional organic carbon or other electron donors to sustain the cycle. Examining the microbial taxonomic and functional potential of such systems is critical to understanding the links between aerobic and anaerobic biogeochemical feedbacks (Johnston *et al.*, 2009).

Here, we evaluated the metabolic potential of the organisms in this community using genomic and metagenomic sequencing. High-throughput DNA sequencing provides opportunities to obtain nearly complete genomes in systems of moderate complexity, or in systems with low strain diversity of a specific ecotype (e.g., Tyson *et al.*, 2004; Iverson *et al.*, 2012). In accordance with this, we were able to analyze several nearly complete genomes. We confirmed that strain ML1 (*Chromatiaceae*) is the dominant PSB in the Mahoney Lake chemocline and that its closest taxonomic relatives belong to the genus *Thiohalocapsa*. In addition, there is a single major taxon of Epsilonproteobacteria (*Sulfurimonas* sp.); eight putative sulfate-reducing Deltaproteobacteria; other phototrophs including Cyanobacteria, Alphaproteobacteria, and GSB; and a diverse array of other organisms from the phyla Firmicutes, Mollicutes, Bacteroidetes, Actinobacteria, and Deinococcus. The results suggest that a high degree of functional redundancy exists within a system that nonetheless possesses limited taxonomic diversity within the major populations. The data also point to specific interpretations about the energy balance of sulfide-driven autotrophy and – via the taxonomic placement of genes for okenone synthesis – address the continuity between planktonic and benthic low-oxygen ecosystems.

MATERIALS AND METHODS

Chromatiaceae strain ML1: DNA Extraction, genomic sequencing, and annotation

A sample for enrichment and isolation of strain ML1 was collected from the chemocline of Mahoney Lake at a depth of 7-m on October 1, 1988 (Fig. 1A; J. Overmann, unpubl. data), and has been maintained since as an enrichment culture (Fig. 1B) using Pfennig's medium as described previously (Overmann & Pfennig, 1989). Genomic DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) protocol for bacterial DNA isolation of the Joint Genome Institute [http://my.jgi.doe.gov/general/]. Purified DNA was sequenced with

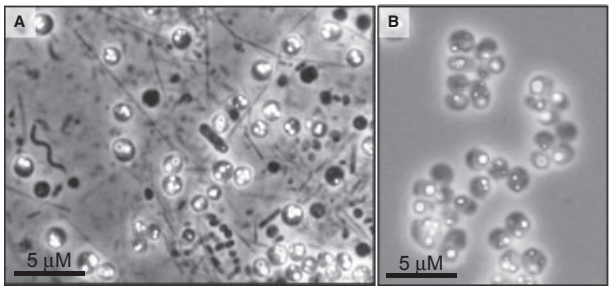

Fig. 1 Phase contrast photomicrograph of the Mahoney Lake chemocline community (A) and of strain ML1 in culture with visible intracellular sulfur globules (B).

a 454 pyrosequencer (GS FLX+; Roche, Branford, CT, USA) in the Schuster Lab at The Pennsylvania State University. A total of 278 972 211 bases in 750 635 reads, with an average read length of 372 bp were generated and assembled by Newbler assembler version 2.6 (Roche) into 867 contiguous reads (contigs) of 500 bp or larger with an average read depth of ~ 40×. Contigs belonging to strain ML1 were assigned by their GC-content and verified with BLAST (Altschul *et al.*, 1990); 137 contigs (6 305 354 bp in total) with content of >53% were assigned to strain ML-1 in this way (Table 1). Contigs were annotated using RAST (http://rast.nmpdr.org; Aziz *et al.*, 2008).

Environmental sample collection, DNA extraction, and metagenomic sequencing

Samples were collected from the phototrophic plate at 7 m from Mahoney Lake, British Columbia (49°17′N, 119°35′W) in July 2008. Samples were frozen on dry ice for transport to Harvard University, where they were stored at −80 °C until further processed. Samples were thawed and centrifuged, and DNA was extracted from the resulting pellet with an e.Z.N.A SP Plant Maxi Kit (Omega Biotek, Norcross, GA, USA) according to the instructions of the manufacturer. The yield and quality of the extracted DNA was assessed using gel electrophoresis and spectrophotometry (Nanodrop, Wilmington, DE, USA). Fragmentation and library preparation were performed by the North Carolina State University Genomic Sciences Laboratory. Paired-end 150-bp Illumina (HiSeq 2500; Illumina, San Diego, CA, USA) sequencing was performed at the Harvard Center for Systems Biology and resulted in ~300 000 000 high-quality reads.

Table 1 Statistics for the strain ML1 draft genome and the ML-Gamma and ML-Epsilon genomic bins

	Strain ML1	ML-Gamma	ML-Epsilon
Scaffolds	137	485	72
Longest (bp)	332 978	105 972	114 097
General information			
Total bp	6 305 354	5 442 835	2 742 932
Average length (bp)	46 024	12 597	29 155
N50 (bp)	114 369	17 120	38 096
Characteristics			
G + C content	68.3	65.9	38.6
Protein coding genes	5664	5433	2732
rRNA genes			
5S	1	1	0
16S	1	1	0
23S	1	1	0
tRNA genes	42	57	48

Metagenome assembly, binning, and annotation

Illumina sequencing reads were trimmed with Trimmomatic 0.20 (Lohse *et al.*, 2012). Only sequences with at least 50 base pairs in both the forward and reverse direction were retained and assembled into contigs with IDBA-UD 1.09 (Table S1), using eight threads with default parameters (Peng *et al.*, 2012). Coverage was determined by aligning raw reads to contigs with BWA 0.5.9 (Li & Durbin, 2009).

The assembled contigs were binned using emergent self-organizing maps (ESOM) based on tetranucleotide frequency, which resulted in clusters corresponding to taxonomically sorted tetranucleotide usage patterns (Dick *et al.*, 2009). A custom Python script (available at http://github.com/bovee/Ochre) was used to calculate tetranucleotide frequency of all contigs ≥2500 bp. Corresponding reverse-complement tetranucleotides were combined as described (Dick *et al.*, 2009). Contigs were split into 5000-bp segments, clustered into taxonomic groups [or 'genomic bins' (Voorhies *et al.*, 2012)] by tetranucleotide frequency and visualized with Databionic-ESOM (http://databionic-esom.sourceforge.net) – again using parameters from Dick *et al.* (2009) (Fig. S1). Bins were inspected for coverage. (Multimodal distribution could indicate the bin contained scaffolds derived from multiple genomes, requiring that the bin be further split, but no such instances were found.) Following manual inspection and further curation by BLAST, phylum-level taxonomic assignment was performed using Phyloshop (Shah *et al.*, 2010) and MEGAN (Huson *et al.*, 2011). Contigs larger than 2500 bp were annotated with an in-house annotation pipeline as described in the supporting online material (SOM).

The two highest-coverage bins in the metagenome, a Gammaproteobacterium referred to here as ML-Gamma and an Epsilonproteobacterium referred to as ML-Epsilon, plus four Deltaproteobacteria bins (ML-Delta 1-4) (Figs S1 and S2), were selected for in-depth characterization and taxonomic assignment of their predicted genes. For the ML-Gamma and ML-Epsilon bins, paired reads mapping to scaffolds from each bin were reassembled

using Velvet (Zerbino & Birney, 2008) as previously described (Hug *et al.*, 2013). Scaffolds of each re-assembly were annotated as described in the SOM. Genome completeness was evaluated using a suite of 76 genes selected from a set of single-copy marker genes that show no evidence for lateral gene transfer (Sorek *et al.*, 2007; Wu & Eisen, 2008) (Tables S2 and S3). The ML-Gamma genomic bin was annotated by RAST for comparison to the strain ML1 (*Chromatiaceae*) genome. The number of genomes per bin assigned to the Deltaproteobacteria was estimated using 35 single-copy orthologous groups (Raes *et al.*, 2007; Handley *et al.*, 2012; Table S4). Genome coverage was estimated by assuming that the genome size of each phylotype was approximately the same as its closest relative (Whitaker & Banfield, 2006; Jones *et al.*, 2012).

Taxonomic assignments and phylogenetic analyses

16S rRNA sequences often fail to assemble into larger contigs and scaffolds in metagenomic studies, and independent assemblies of these genes [e.g., Phyloshop (Shah *et al.*, 2010) or EMIRGE (Miller *et al.*, 2011)] are difficult to assign to bins separated by genomic signatures such as %GC-content or tetranucleotide frequency. To overcome these obstacles, single-copy ribosomal proteins were analyzed as described in the SOM (Table S5) to make genus-level assignments of the ML-Gamma genomic bin and the ML-Epsilon genomic bin. Such analyses yield resolution comparable to that for 16S rRNA phylogenetic trees (Hug *et al.*, 2013).

The phylogenetic position of bacterial 16S rRNA genes was evaluated by approximate likelihood-ratio tests (Anisimova & Gascuel, 2006) as implemented in PhyML. Sequences were aligned and the best evolutionary model was determined using JMODELTEST (version 2.1.1, Darriba *et al.*, 2012). Maximum-likelihood reconstructions used the General Time Reversible substitution model and gamma-distributed rate variation with a proportion of invariable sites as recommended by JMODELTEST. Phylograms were rate-smoothed using the multidimensional version of Rambaut's parameterization as implemented in PAUP v. 4.0 (Swofford, 2001) as previously described (Meuser *et al.*, 2013). Methods for gene-specific phylogenetic analyses (e.g., *soxB*, *cruO*; Table S6) are provided in the SOM.

Nucleotide sequence accession numbers

Metagenomic sequence data are deposited at CAMERA (http://camera.calit2.net/), and the assembled sequences can be accessed via IMG/M (http://img.jgi.doe.gov). Metagenome bin sequences – ML-Gamma and ML-Epsilon – are deposited at DDBJ/EMBL/GenBank under the accession numbers AZMQ00000000 and AZMQ00000000, respectively; and the versions described in this paper are AZMQ01000000 and AZMQ010000000, respectively. The draft genome sequence of *Thiohalocapsa* sp. strain ML1 (*Chromatiaceae*) is deposited at DDBJ/EMBL/GenBank under the accession JABX00000000. The version described in this paper is version JABX01000000. Gene IDs reported in Table S6 represent the assembled gene sequences available on the IMG/M Web site.

RESULTS

Genomic sequencing of the highly enriched culture of strain ML1 resulted in a draft genome of strain ML1, a *Chromatiaceae* sp. Contigs of the draft genome of strain ML1 were easily resolved from a contaminant organism belonging to the class Mollicutes by %GC (68% vs. ~30%) and verified by BLAST. The draft genome assembled into 137 scaffolds containing 6.3 Mbp (Table 1).

De novo assembly of the metagenomic data from 7 m water depth resulted in 452 140 contigs containing ~ 640 Mbp. The vast majority of contigs could be assigned to bacteria, while smaller numbers of eukaryotic, archaeal, and viral sequences were recovered (Fig. 2A). Most of the bacterial contigs (>60%) were affiliated with Proteobacteria, but sequences affiliated with Firmicutes, Bacteroidetes, and Actinobacteria were also abundant (Fig. 2B). Tetranucleotide frequency analysis of the *de novo* metagenomic assembly resolved 26 distinct taxonomic bins (Fig. S1), including well-defined genomic bins for a Gammaproteobacterium (ML-Gamma) and an Epsilonproteobacterium (ML-Epsilon). These two organisms represent the dominant members associated with the oxidative arm of the sulfur cycle. Four distinct bins of sulfate-reducing Deltaproteobacteria (ML-Delta), including *Desulfobacterales* and *Desulfovibrionales*, were also resolved, and these bins collectively contained at least eight genomes (Fig. S2). As observed in other studies employing similar binning strategies (e.g., Handley *et al.*, 2012), the coverage, GC-content, and phylogenetic similarity of scaffolds within the Deltaproteobacteria bins precluded further resolution of individual genomes. In contrast, the Gamma- and Epsilonproteobacteria bins each contained a single genome. We also recovered six low coverage bins affiliated with the *Rhodobacteriales* of the Alphaproteobacteria, three bins of Actinobacteria and Bacteroidetes, two other (non-phototrophic) Gammaproteobacteria bins (*Xanthomonadales* and *Thiotricales*), and single bins affiliated with Betaproteobacteria (*Burkholderiales*), Cyanobacteria, Spirochetes, and Mollicutes, (Fig. S1). Based on %GC and BLAST results, the Mollicutes bin recovered from the metagenome is not the same Mollicutes organism present in the co-culture with strain ML1 (data not shown). These results reveal a diverse capacity for sulfur and carbon cycling in the Mahoney Lake chemocline.

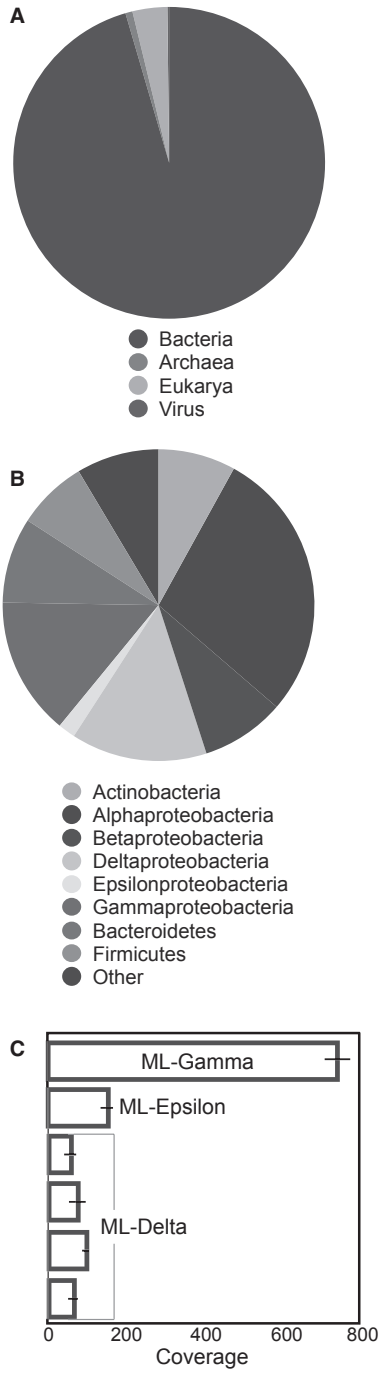

Fig. 2 Taxonomic affiliation of all sequences in the 7-m metagenome and coverage of genome bins. (A) Number of sequences affiliated with each domain. (B) Number of sequences assigned at the phylum level (except the Proteobacteria which are represented by class). (C) Genomic bins distinguished by read coverage. Other indicates all phyla represented by <800 sequences.

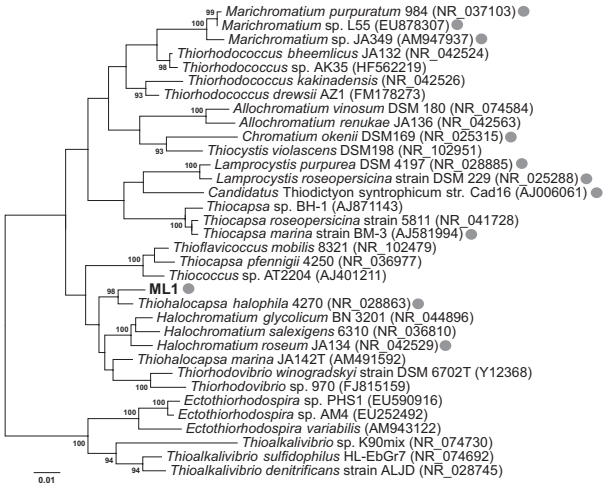

Fig. 3 Maximum-likelihood-based phylogenetic 16S rRNA gene tree of closely related Gammaproteobacteria and strain ML1 (*Chromatiaceae*). Accession numbers are provided in parentheses. Gray circles indicate species that synthesize okenone. Bootstrap support values based on 1000 bootstrap samplings >90 are noted.

Here, we will focus on genomic insights as they relate to sulfur and carbon cycling catalyzed by the high-coverage Gammaproteobacterium, the Epsilonproteobacterium, and the members of the Deltaproteobacteria. Together, these taxa are sufficient to demonstrate a complete oxidative, reductive, and phototrophic sulfur cycle. We also discuss the correspondence between the high-coverage Gammaproteobacterium and isolate strain ML1, and we address implications for the presence of the carotenoid pigment, okenone, in the geologic record.

Oxidative sulfur cycle

Strain ML1 (*Chromatiaceae*) and the ML-Gamma genomic bin

The highest-coverage organism in the metagenome, ML-Gamma (Fig. 2C), contains a 16S rRNA gene identical to strain ML1 (Coolen & Overmann, 1998; EMBL acc. no. AJ006212). Phylogenetic analysis of 19 concatenated phylogenetic marker genes (Table S5) indicates that the ML-Gamma genome bin is strain ML1 or a very closely related ecotype (Fig. S3). Alignment of single-copy genes between strain ML1 and the ML-Gamma bin (Table S2) shows >99% identity across these phylogenetic markers. The ML-Gamma genome bin is similar in size (5.7 Mbp) to the draft genome (6.3 Mbp) of strain ML1 and contains 73 of these 76 marker genes (Table S2), indicating that it is >90% complete.

Taxonomic assignment places strain ML1 into the order *Chromatiales* and the family *Chromatiaceae*. In contrast to the initial assignment based on morphological and physiological characteristics, our 16S rRNA gene sequence analysis indicates that this strain is most closely related to *Thiohalocapsa* spp. and *Halochromatium* spp. (Fig. 3). The 16S rRNA gene of strain ML1 is 98% identical to that of *Thiohalocapsa halophila* 4270^T (DSM 6210^T), which is its closest cultured relative. In contrast, phylogenetic analysis

based on concatenated sequences of phylogenetic marker genes shows that strain ML1 is most closely affiliated with *Thiorhodovibrio* spp., including *Thiorhodovibrio* sp. 970 and the Mahoney Lake shoreline isolate *Thiorhodovibrio winogradsky* (Overmann *et al.*, 1992) (Fig. S3); however, this analysis is restricted to available complete genomic sequences, which currently includes no members of the genera *Thiohalocapsa* and *Halochromatium*.

Strain ML1 and the ML-Gamma metagenomic bin both encode the genes necessary for photoautotrophic growth utilizing reduced sulfur compounds as the electron source for carbon dioxide reduction. These genomes contain a *puf* operon encoding a type-2 photochemical reaction center comprised of the subunits PufL, PufM, and PufH, which are identical in arrangement and sequence to the previously published *puf* operon of the isolate (Tuschak *et al.*, 2005); bacteriochlorophyll *a* biosynthesis genes; and genes (e.g., *csoS*) for alpha-carboxysomes. Both genomes contain *cbbLS* and *cbbM* genes, which encode Forms I and II of RuBisCO, the key enzyme in the Calvin–Benson–Bassham (reductive pentose phosphate) cycle. Genes encoding the large and small subunit of RuBisCO form I, *cbbL* and *cbbS*, are clustered with *cso* genes encoding carboxysome shell proteins; while the form II gene *cbbM* is located elsewhere in the genome. The CbbL and CbbM sequences of strains ML1 and the ML-Gamma genome are closely related to those of the species '*Candidatus* Thiodictyon syntrophicum' strain Cad16 from Lake Cadagno (Peduzzi *et al.*, 2012) (Fig. S4), which preferentially expresses Form I RuBisCO when CO_2 assimilation rates are high (Storelli *et al.*, 2013). Consistent with the observation of gas vesicles both in the isolate and in samples collected from the chemocline (Overmann *et al.*, 1991), we observed *gvp* genes which encode gas vesicle proteins. Both genomes also encode a cbb_3-type heme-copper cytochrome oxidase, which is capable of maintaining catalytic activity under micro-oxic conditions (Pitcher & Watmough, 2004), as well as genes for a *hox*-type, bi-directional [NiFe]-hydrogenase and succinate dehydrogenase. Genes encoding a Type-1 NADH dehydrogenase (*nuo*), Mo-dependent nitrogenase (*nifHDK*) (Fig. S5), and an Rnf-electron transport complex (*Rhodobacter* nitrogen fixation, *rnf*-encoded) were also identified in both genomes. Rnf complexes are widely distributed and are thought to mediate electron transfer between NADH and ferredoxin (Biegel *et al.*, 2011), and they may also play a role in nitrogen fixation (Jeong & Jouanneau, 2000).

The data also confirm that this taxon encodes all enzymes necessary for biosynthesis of the carotenoid okenone, an abundant compound in the Mahoney Lake chemocline particulates, along the shoreline, and in sediment cores (Overmann *et al.*, 1991) (Fig. 4). These data include genes encoding an *O*-methyltransferase (CrtF), the CrtY and CrtU proteins necessary for synthesis of the

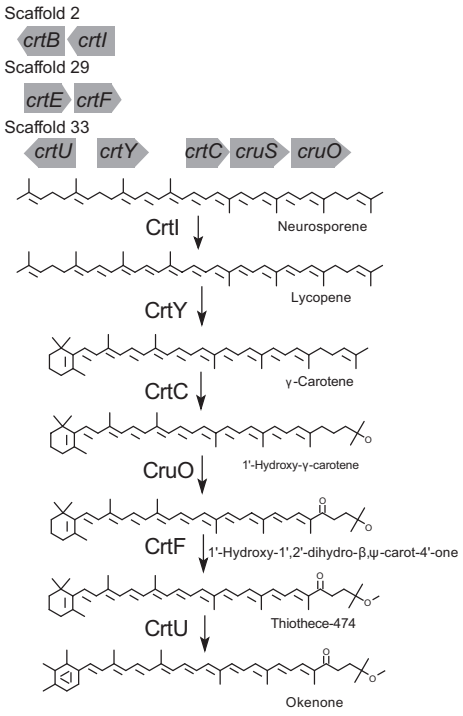

Fig. 4 Predicted pathway for okenone synthesis of strain ML1 and ML-Gamma based on genes identified in the genome and genome bin, respectively. Scaffold numbers refer to the location of predicted genes in the draft genome of strain ML1. Proteins and the reactions they catalyze in the pathway for the biosynthesis of okenone, from Vogl & Bryant, 2011, are summarized.

χ-ring of okenone, and the unique CruO 4/4′ ketolase protein (Vogl & Bryant, 2011, 2012) (Fig. S6). Importantly, the genes encoding CruO and the associated CruS enzyme in the ML-Gamma genome bin are the only copies of these diagnostic genes detected in the entire metagenome, establishing that this PSB taxon is probably the only source of okenone in the chemocline.

The genomes of strain ML1 and ML-Gamma encode thiosulfate oxidation enzymes (Sox), a flavocytochrome *c*-sulfide dehydrogenase (Fcc), and a D-type sulfide:quinone oxidoreductase (Sqr) (Fig. 5, Fig. S7) (Gregersen *et al.*, 2011). SoxA, B, X, Y, and Z comprise a multi-enzyme complex of SoxXA, a heterodimeric *c*-type cytochrome; SoxB; and SoxYZ, a heterodimeric thiosulfate-binding protein necessary for oxidation of thiosulfate to sulfate. As in other purple sulfur bacteria, no *soxCD* genes were found in either the isolate genome or the genome bin. In other *Chromatiaceae* that lack *soxCD* – for example, *Allochromatium vinosum* – sulfur/polysulfide globules form in the periplasm as oxidation intermediates (Hensen *et al.*, 2006; Frigaard & Dahl, 2009; Dahl *et al.*, 2013). In *Alc. vinosum*, reverse dissimilatory sulfite reductase (DsrAB) and other related Dsr proteins – including the transmembrane complex DsrMKJOP, the DsrEFH complex, and DsrC – are necessary for oxidation of the

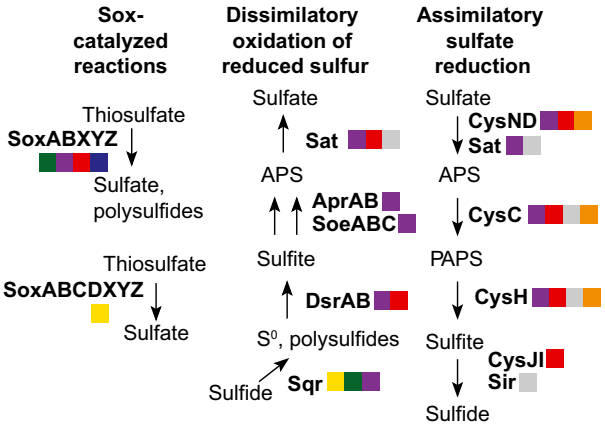

Fig. 5 Predicted oxidative sulfur cycle in the Mahoney Lake chemocline based on genes identified in the metagenome. Colors indicate the phylogenetic affiliation of the predicted proteins. Red, Alphaproteobacteria; Yellow, Epsilonproteobacteria; Purple, Gammaproteobacteria; Green, green sulfur bacteria; Gray, Cyanobacteria; Blue, Betaproteobacteria; Orange, Bacteroidetes. Abbreviations: APS, Adenosine-5′-phosphosulfate; PAPS, 3-Phosphoadenosine-5′-phosphosulfate.

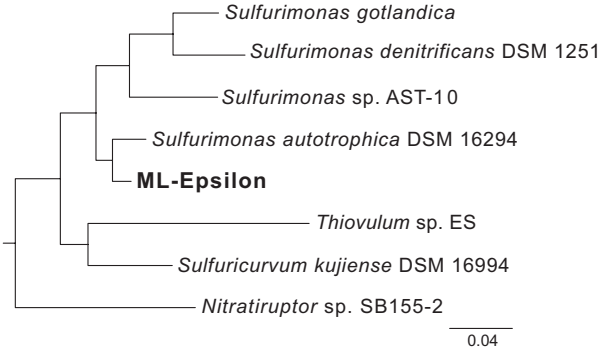

Fig. 6 Maximum-likelihood phylogenetic tree of 14 concatenated single-copy ribosomal proteins (Table S5) showing the taxonomic placement of the ML-Epsilon genomic bin. Bootstrap support values based on 1000 bootstrap samplings were >85 for each node.

stored sulfur to sulfite (Dahl *et al.*, 2008; Oliveira *et al.*, 2008; Grein *et al.*, 2010). The DsrEFH complex, involved in sulfur transfer reactions, is encoded in genomes of sulfur-oxidizing prokaryotes that also encode DsrAB but is absent in sulfate-reducing organisms (Sander *et al.*, 2006; Grimm *et al.*, 2008). Genes encoding the reverse Dsr proteins, including DsrEFH, are present in strain ML1 (and ML-Gamma), and their gene context is similar to the genomic context that is observed in *Alc. vinosum* (Grein *et al.*, 2010).

Genes encoding homologues of key enzymes involved in dissimilatory oxidation of reduced sulfur and assimilatory sulfate reduction are present (Fig. 5), including an adenosine phosphosulfate reductase (APS reductase, ApsAB) and a sulfate adenylyltransferase (ATP-sulfurylase, Sat). In some PSB, indirect oxidation of sulfite in the cytoplasm results from the activity of APS reductase and ATP-sulfurylase; however, these enzymes are not required for sulfite oxidation and are not universally found in sulfur-oxidizing prokaryotes (Dahl *et al.*, 2013). The genomes also encode homologues of the SoeABC sulfite-oxidizing enzyme complex, which is believed to oxidize sulfite presumably by transferring electrons to the electron transport chain via the quinone pool (Dahl *et al.*, 2013), as well as enzymes involved in assimilatory sulfate reduction including adenylyl sulfate kinase (CysC), phosphoadenosine phosphosulfate reductase (CysH), and an NADPH-dependent sulfite reductase (CysJI) (Fig. 5).

The ML-Epsilon genomic bin

The ML 7-m metagenome contains a well-defined genomic bin identified as an Epsilonproteobacterium closely related to *Sulfurimonas autotrophica* (Fig. 6), a chemolithoautotrophic, sulfur-oxidizing aerobe that can use elemental sulfur, sulfide, and thiosulfate as electron donors (Inagaki *et al.*, 2003). Based on the presence of phylogenetic marker genes, the assembled genome is estimated to be >90% complete, and it is similar in size (~2.7 Mbp) (Table 1) to that of several closely related species, including the genome of *S. autotrophica* (~2.1 Mbp). Similar to characterized chemolithoautotrophic Epsilonproteobacteria, the ML-Epsilon genome encodes the enzymes necessary for carbon fixation via the reverse TCA (rTCA) cycle, including ATP-dependent citrate lyase (*acl*), pyruvate: ferredoxin oxidoreductase (*porAB*), and 2-oxoglutarate: ferredoxin oxidoreductase (*oorAB*) (Campbell *et al.*, 2006), but does not contain RuBisCO genes (*cbbLS* or *cbbM*). Unlike *S. autotrophica,* the ML-Epsilon genome encodes enzymes for the reduction of nitrate to N_2, including a periplasmic nitrate reductase (NapAB), a cytochrome cd_1-dependent nitrite reductase (NirS), a nitric oxide reductase (NorBC), and nitrous oxide reductase (NosZ). These genes, together with the *sox* and *sqr* genes, suggest that nitrate-dependent sulfide oxidation can occur under anoxic conditions (Simon & Klotz, 2013). Similar to other *Sulfurimonas* spp., the ML-Epsilon genomic bin encodes a Mo-dependent nitrogenase and an ammonium transporter (Amt). It also contains cytochrome cbb_3 oxidase and cytochrome *bd* quinol oxidase genes, both of which are high-affinity terminal oxygen reductases capable of functioning under micro-oxic conditions; formate dehydrogenase (*fdhABC* genes); and two [NiFe]-hydrogenases, one cytoplasmic and one membrane-bound – suggesting this organism may use H_2 and formate as electron donors in addition to sulfide.

Reflecting its role in the sulfur cycle, the genome contains a B-type sulfide:quinone oxidoreductase (Sqr) (Fig. S7) (Gregersen *et al.*, 2011), which catalyzes the oxidation of sulfide to polysulfide, and *soxCD* genes, which indicate that the organism is capable of oxidizing

thiosulfate to sulfate (Fig. 5). The genome also encodes a membrane-bound polysulfide reductase (*psr*-encoded), which may enable the use of polysulfide as an electron acceptor (Hedderich *et al.*, 1998); a sulfite cytochrome *c* oxidoreductase (Sor), possibly involved in the inorganic disproportionation of reduced sulfur compounds (Finster, 2008); and a rhodanese-related sulfurtransferase presumably involved in the oxidation of thiosulfate (Ghosh & Dam, 2009).

Other organisms putatively involved in the oxidative sulfur cycle

The 7-m metagenome contains multiple copies of key enzymes involved in sulfur oxidation that are assigned to other taxonomic bins. These include genes encoding sulfide:quinone oxidoreductase (Sqr), flavocytochrome *c* (Fcc), components of dissimilatory sulfite reductase (DsrAB, DsrEFH, DsrC, and the DsrMKJOP complex), the Sox multi-enzyme complex, and components of assimilatory sulfate reduction (CysND, CysC, CysH, CysJI, Sat, and Sir) (Fig. 5; Table S7). The *sox*-encoded enzymes of thiosulfate oxidation are most abundant among these, while six copies each of homologs of DsrE, F, and H, specific to sulfur-oxidizing organisms are present. Full-length SoxB sequences affiliated with the Alpha-, Beta-, and Gammaproteobacteria were recovered from other tetranucleotide-resolved genomic bins (Fig. 7). More putative sequences for SoxA and SoxB were present (31 and 33 copies, respectively) than the *sox*-encoded proteins C, D, X, Y, Z (Table S7), and the majority are affiliated with Alphaproteobacteria. Several *sox* and *sqr* genes affiliated with GSB, which use the Sox system for thiosulfate oxidation (Ogawa *et al.*, 2008; Gregersen *et al.*, 2011), were also observed (Table S7), despite the low number of reads assigned to the phylum Chlorobi. The translated *sqr* sequences cluster near the SqrD-type of *Chlorobaculum tepidum* TLS (Gregersen *et al.*, 2011; Holkenbrink *et al.*, 2011) (Fig. S7; Table S8).

Reductive arm of the sulfur cycle

ML Deltaproteobacteria community genomic bins

The Mahoney Lake chemocline contains multiple taxa of Deltaproteobacteria, including at least eleven distinct genotypes (Fig. S8A) based on ribosomal protein S3 sequences (Hug *et al.*, 2013). At least six genomes of *Desulfurobacteriales* spp. (present in genomic bins designated Delta 1, 3, and 4) and at least two *Desulfovibrionales* spp. genomes (genomic bin designated Delta 2) (Table S9, Figs S1 and S2) could be resolved. The high degree of phylogenetic similarity, GC-content, and coverage among the Deltaproteobacteria spp. precludes the resolution of single genomes. The recovery of multiple sulfate-reducing bacteria (SRB) genomes across several tetranucleotide bins indicates organismal redundancy is also present within the reductive arm of the sulfur cycle.

The genomic bins assigned to the *Desulfurobacteriales* and *Desulfovibrionales* encode the enzymes necessary for dissimilatory sulfate reduction, including a sulfate adenylyltransferase (ATP-sulfurylase, Sat), an APS reductase (AprAB), and the dissimilatory sulfite reductase complex (DsrAB, DsrC, and DsrMKJOP), which catalyzes the reduction of SO_3^{2-} to S^{2-} (Fig. S8B). The genomic bins also encode Mn-dependent, inorganic pyrophosphatase (PpaC) and abundant ferredoxins (predominantly Ferrodoxin I). The genomes contain homologues of D-lactate dehydrogenase (gene *ldhA*) and pyruvate dehydrogenase (gene *pdh*) for conversion of lactate to acetyl-CoA, as well as carbon monoxide dehydrogenase/acetyl-CoA synthetase (CODH/ACS). Many acetotrophic SRBs employ a CODH/ACS pathway to cleave acetyl-CoA, and yet the presence of CODH/ACS genes in SRBs closely related to autotrophic species (i.e., *Desulfovibrio* spp.; Fig. S8A) also suggests at least facultative autotrophy may occur (Schauder *et al.*, 1989). The ML-*Desulfovibrionales* genomic bins also encode fumarate reductases, indicating that these organisms may be capable of fumarate respiration; a periplasmic [FeFe]-hydrogenase (HydA); and heterodisulfide reductase-like proteins, specifically HdrA- and HdrD-related proteins, which are thought to be involved in sulfite reduction through DsrC or reduction of the menaquinone pool (Pereira *et al.*, 2011). The SRB genomic bins also encode Mo-dependent nitrogenases (*nifHDK*).

Other organisms putatively involved in the reductive sulfur cycle

Genes from other species involved in reducing sulfur compounds include 21 unique copies of *dsrD*. Although the exact function of DsrD remains unknown, it is postulated to be involved in sulfite reduction (Grein *et al.*, 2013) and is typical of SRB, but it is not found in sulfur-oxidizing organisms (Grimm *et al.*, 2008). The metagenome also contains tetrathionate reductase genes (*ttr*), which catalyze the reduction of tetrathionate to thiosulfate and are widely distributed in the Enterobacteriaceae and other facultative anaerobes (Zopfi *et al.*, 2004). Dimethyl sulfoxide (DMSO) reductase (DmsA), also common in anaerobic heterotrophic bacteria, was identified. The majority of *ttr* and *dmsA* sequences were affiliated with the Firmicutes, Deltaproteobacteria, and the Alpha- and Gammaproteobacteria (Table S7).

Phylogenetic diversity of *dsr*

The 7-m metagenome contains eleven distinct copies of *dsrAB*, encoding dissimilatory sulfite reductase, the enzyme necessary for sulfite reduction or sulfur oxidation (Fig. 8). Concatenated amino acid sequence alignments of full-

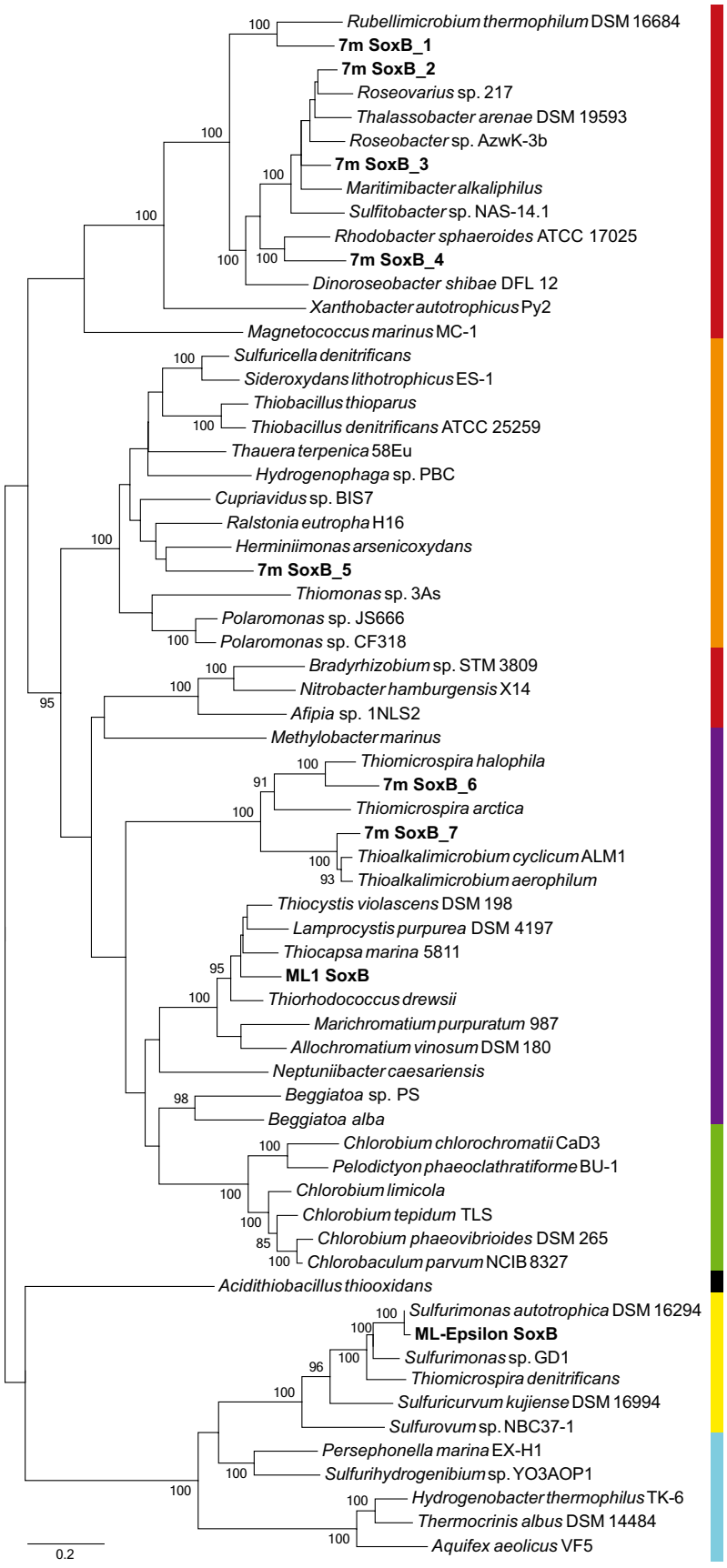

Fig. 7 Phylogenetic diversity of SoxB in the Mahoney Lake chemocline. Maximum-likelihood phylogenetic tree constructed from translated SoxB sequences identified in the ML 7-m metagenome. Red, Alphaproteobacteria; orange, Betaproteobacteria; purple, Gammapro teobacteria; green, green sulfur bacteria; black, Acidithiobacillia; yellow, Epsilonproteobacteria; blue, Aquificales. The strain ML1 genome and ML-Gamma genomic bin contain identical SoxB sequences represented here as ML1. Abbreviations: SRB, sulfate-reducing bacteria; SOB, sulfur-oxidizing bacteria. Bootstrap support values based on 1000 bootstrap samplings ≥85 are shown for each node.

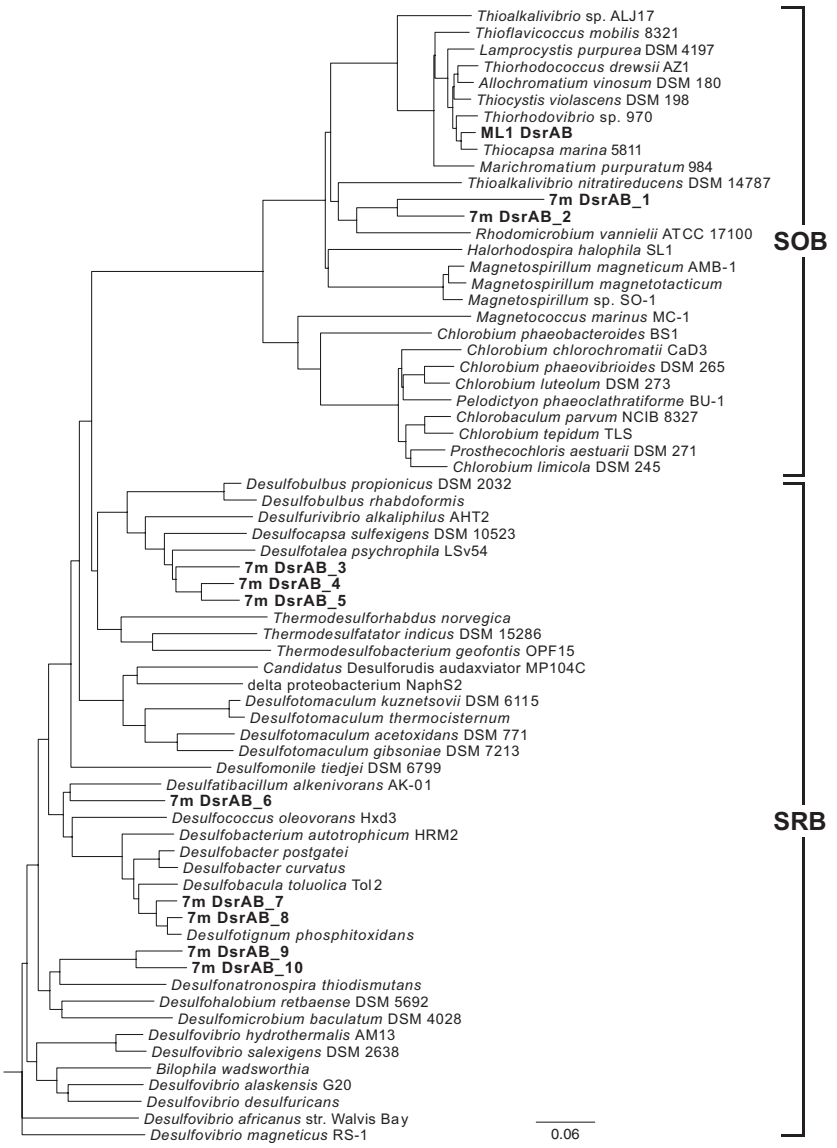

Fig. 8 Phylogenetic diversity of dissimilatory sulfate reductase (Dsr) in the Mahoney Lake chemocline. Maximum-likelihood phylogeny calculated from a concatenated amino acid sequence alignment of DsrA and DsrB sequences recovered from the ML 7-m metagenome and representative sequences from the Deltaproteobacteria and sulfur-oxidizing members of the Alpha- and Gammaproteobacteria. DsrA and DsrB sequences of strain ML1 and ML-Gamma are identical and represented by ML1. Abbreviations: SRB, sulfate-reducing bacteria; SOB, sulfur-oxidizing bacteria.

length alpha and beta subunits (DsrA and DsrB, respectively) confirm the presence of multiple sulfate-reducing Deltaproteobacteria-affiliated sequences and several sulfur-oxidizing Alpha- and Gammaproteobacteria-affiliated sequences. Several of these SRB-affiliated sequences are not represented in the genome bins discussed above and presumably are derived from other less abundant taxa in the system. No *dsrA* or *dsrB* genes affiliated with GSB were recovered despite the observation that nearly all GSB contain *dsr* (Frigaard & Bryant, 2008). This is most likely due to the low abundance of reads assigned to GSB in the 7-m metagenome.

Phylogenetic diversity of RuBisCO

In addition to the ML-Gamma *cbbL* and *cbbM* sequences, the 7-m metagenome contains fourteen full-length genes encoding ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Of these, five group with the Type IV RuBisCO-like proteins (RLP), which apparently do not catalyze RuBP carboxylation or oxygenation (Hanson & Tabita, 2003; Tabita *et al.*, 2007) (Fig. 9, Fig. S9). In the GSB *Chlorobaculum tepidum*, a RLP has been implicated in sulfur metabolism (Hanson & Tabita, 2003), while in some Alphaproteobacteria, RLP are involved in 5-methylthioadenosine-dependent growth (Tabita *et al.*, 2007). The remaining sequences all branch with characterized RuBisCO proteins, indicating they are involved in CO_2 assimilation *in situ*. In addition to the Type I and Type II RuBisCO identified in the ML-Gamma genomic bin, the metagenome contains three sequences affiliated with Type I RuBisCO (similar to *Thioalkalvibrio* spp. and members of the phyla Cyanobacteria and Chloroflexi) and two affiliated with Type II RuBisCO (similar to sequences of *Sulfuricella denitrificans*, a

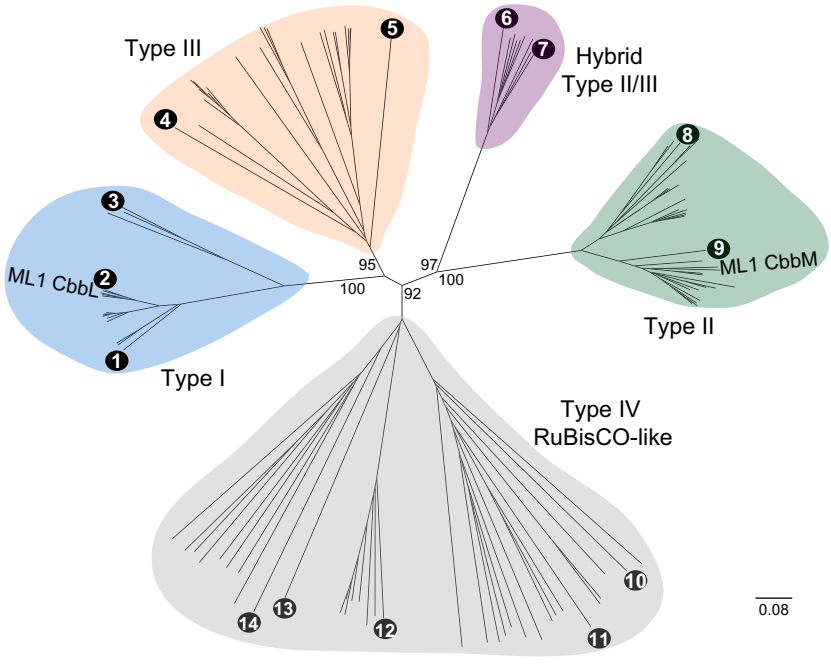

Fig. 9 Phylogenetic diversity of RuBisCO in the Mahoney Lake chemocline. Maximum-likelihood phylogenetic trees constructed from CbbL and CbbM sequences mined from the ML 7-m metagenome and the NCBI and IMG/M database. Numbers indicate full-length sequences mined from the ML 7-m metagenome (Table S6) and the full tree is shown in Fig. S9. CbbL and CbbM sequences of strain ML1 and ML-Gamma are identical and represented by ML1. Bootstrap support values based on 1000 bootstrap samplings are shown for each node.

sulfur-oxidizing Betaproteobacterium, and *R. rubrum*) (Fig. S9). Two additional sequences branch with the methanogen Type III RuBisCO and two sequences group together with the novel bacterial–archaeal hybrid TypeII/III sequences (Fig 8), which are putatively functionally analogous to Type III (Tabita *et al.*, 2007); these enzymes are thought to fix CO_2 and contribute to adenosine monophosphate (AMP) recycling (Wrighton *et al.*, 2012).

DISCUSSION

Identification of the dominant PSB in Mahoney Lake

The enrichment culture strain ML1 (*Chromatiaceae*; Overmann *et al.*, 1991) is very closely related to the highest-coverage population in the 7-m metagenome, indicating that strain ML1 is representative of the major PSB population. This PSB is believed to be the most abundant organism in the Mahoney Lake chemocline (Overmann *et al.*, 1991), and the similarity between our metagenome (sampled in 2008) and strain ML1 (enriched in 1989) confirms decadal stability of this population. The initial classification of strain ML1 as *Amoebobacter purpureus* was based on cell morphology, the presence of gas vesicles, okenone production, and its ability to use glucose, pyruvate, and acetate (Overmann *et al.*, 1991). Due to the re-classification of *A. purpureus* as a *Lamprocystis* species (Guyoneaud *et al.*, 1998), strain ML1 has been referred to as '*Lamprocystis purpurea* ML1' (EMBL acc. nos. AJ006212, AY177752, Klepac-Ceraj *et al.*, 2012). However, initial analyses of the 16S SSU rRNA sequence suggested that the isolate had been misclassified (Coolen & Overmann, 1998), an observation bolstered by more recent analyses of the 16S rRNA and the *pufLM* genes, both of which indicated that strain ML1 is affiliated with the genus *Thiohalocapsa* (Tank *et al.*, 2009). Phylogenetic analyses presented here and elsewhere (Coolen & Overmann, 1998; Tank *et al.*, 2009) show this organism is most closely related to marine mat-forming members of the *Chromatiaceae,* such as *Thc. halophila* as well as *Halochromatium roseum* and *Hch. glycolicum*. The 16S rRNA sequences from *Thc. halophila* (formerly *Thiocapsa halophila;* Caumette *et al.*, 1991) and strain ML1 are 98% identical.

Although some characteristics of strain ML1 – for example, the presence of gas vesicles and production of okenone – are also characteristic of *Lamprocystis* spp., strain ML1 exhibits higher salt tolerance than freshwater species (Imhoff, 2005). Moreover, gas vesicles and okenone are not taxonomically diagnostic. Gas vesicles have been observed in multiple genera of *Chromatiaceae*, including *Thiocapsa, Thiodictyon, Thiolamprovum, Thiopedia, Lamprocystis, Lamprobacter*, and *Halochromatium* (Kumar *et al.*, 2007). Okenone production is similarly irregularly distributed; it is mainly found in the gas vesicle containing genera *Thiocapsa*, *Thiopedia*, *Thiodictyon*, *Lamprobacter*, and *Thiolamprovum*, but it is also produced by the halophilic species *Hch. roseum* (Kumar *et al.*, 2007) and the non-gas vacuolate relative of strain ML 1, *Thc. halophila*. Based on the robust 16S rRNA gene analyses presented here, the clade containing strain ML1 and *Thc. halophila* is distinct from other major clades of planktonic PSB (e.g., *Lamprocystis* spp., *Chromatium* spp.) (Fig. 3), including '*Ca*. Thiodictyon syntrophicum' Cad16, an abundant PSB that occurs in the compact chemocline of Lake Cadagno (Storelli *et al.*, 2013). As a result, we

suggest that strain ML1 is a potentially new species within the genus *Thiohalocapsa* and propose that it be renamed *Thiohalocapsa* sp. strain ML1.

A coupled sulfur cycle in a stable planktonic community

Metagenomic sequencing of the Mahoney Lake chemocline indicates this sulfur-rich ecosystem harbors functional redundancy in both the oxidative and reductive arms of the sulfur cycles (Fig. 10A). This functional redundancy could be a key factor in maintaining the apparent stability of the Mahoney Lake chemocline over the past ~30 years, or it may have developed as a result of such stability. The recovery of aerobic taxa such as Cyanobacteria and Epsilonproteobacteria may suggest fine-scale niche separation within the narrow phototrophic plate, the top of which is bounded by the oxic epilimnion. Alternatively, these taxa may represent biomass from the oxygenated layer, which settled and was captured by the dense phototrophic plate. Both Cyanobacteria and Epsilonproteobacteria are capable of living in micro-oxic as well as sulfidic conditions (Jorgensen *et al.*, 1986; Campbell *et al.*, 2006). Conversely, the bottom of the plate intersects the highly sulfidic (35 mM) hypolimnion, an environment also rich in polysulfides, elemental sulfur, and other sulfur intermediates that may be amenable to sulfur disproportionation. Single genome bins (single populations) of sulfur oxidizers recovered in the present study contain multiple pathways for oxidation of sulfur compounds, implying flexibility and/or redundancy in their sulfur metabolism, a genomic characteristic observed in other sulfidic systems (Jones *et al.*, 2012). Isotope values of sulfur species in the chemoclines of other meromictic lakes, such as Fayetteville Green Lake, indicate that multiple processes contribute to sulfur oxidation and that these vary seasonally (Zerkle *et al.*, 2010). A similar seasonal variation may also occur in the

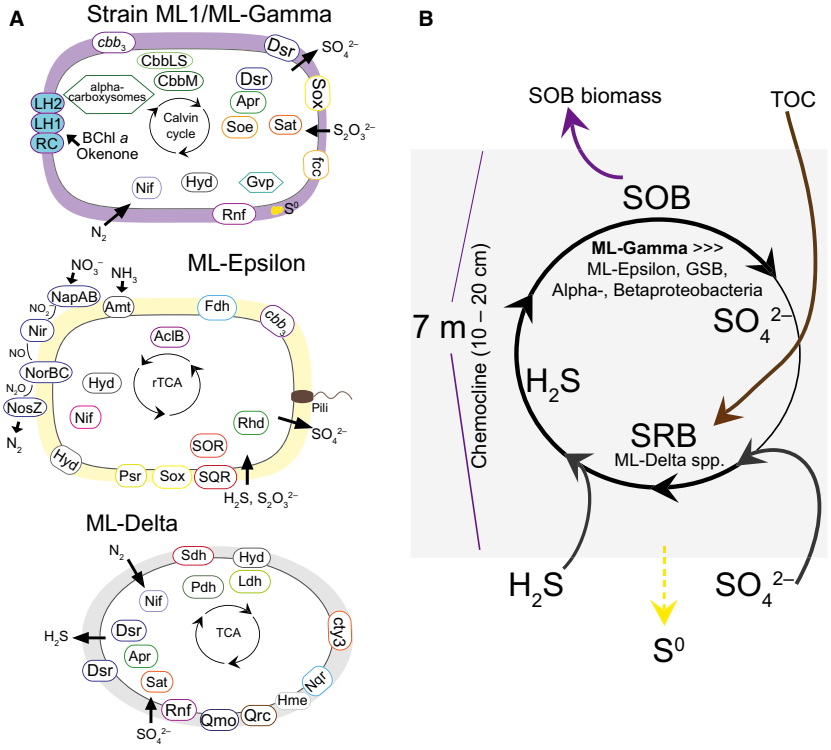

Fig. 10 Genomic models of the strain ML1/ML-Gamma, ML-Epsilon and the ML-Delta spp. (A) and putative model of the sulfur cycle in the Mahoney Lake chemocline (B). The loss of biomass (organic carbon and reduced sulfur, purple arrow) from the chemocline due to rafting, external organic carbon as a significant source of carbon to SRB in the chemocline (TOC, brown arrow) and loss of S^0 to the lower layers of the lake (yellow arrow with dashes) are depicted. Further results and discussion of strain ML1/ML-Gamma, ML-Epsilon, and the ML-Delta spp. are provided in the SOM. AclB, ATP-dependent citrate lyase; Amt, ammonia/ammonium transporter; Apr, adenylyl sulfate (APS) reductase; cbb_3, cytochrome *c* oxidase; Cyt3, cytochrome c_3; Dsr, dissimilatory sulfite reductase; Fdh, formate dehydrogenase; Gvp, gas vesicle proteins; Hyd, hydrogenase; Hme, heterodisulfide reductase-like menaquinol-oxidizing enzyme complex; LH, light-harvesting complex; Ldh, lactate dehydrogenase; NapAB, NO_3^- reductase; Nif, nitrogen fixation; NirS, NO_2^- reductase; NorBC, NO reductase; NosZ, N_2O reductase; Nqr, Na^+-translocating NADH:quinone oxidoreductase; RC, photosynthetic reaction center; Pdh, pyruvate dehydrogenase; Psr, polysulfide reductase; rTCA, reductive TCA cycle; Qmo, quinone-interacting membrane-bound oxidoreductase complex; Qrc, quinone reductase complex; Rhd, rhodanese-related sulfurtransferase; Rnf, Rnf-electron transport complex; Sat, ATP-sulfurylase; Sox, sulfur oxidation proteins; Sdh, succinate dehydrogenase/fumarate reductase; Soe, sulfite-oxidizing enzyme complex; SOR, sulfite oxidoreductase; Sqr, sulfide:quinone oxidoreductase; SOB, sulfur-oxidizing bacteria; SRB, sulfate-reducing bacteria.

chemocline of Mahoney Lake, supporting functional redundancy in sulfur oxidation pathways and a diversity of sulfur-oxidizing species.

Historical data from Mahoney Lake show that the chemocline is relatively stable in both thickness and depth, although seasonal variation (e.g., autumnal die-off) affects the total cell abundance of *Thiohalocapsa* sp. strain ML1 (Overmann *et al.*, 1994). Other anaerobic phototrophic bacteria, including *Thiocapsa* sp. and GSB (Overmann *et al.*, 1991) as well as other autotrophs (e.g., ML-Epsilon; this study) and a large number of Alphaproteobacterial sequences (this study), are observed in the Mahoney Lake chemocline. However, in past studies, *Thiohalocapsa* sp. strain ML1 was responsible for >98% of primary productivity and, on average, 75% of the sulfide oxidation (Overmann *et al.*, 1991, 1996a). In contrast, the narrow chemocline of Lake Cadagno, a meromictic lake in Switzerland, hosts multiple populations of PSB, including *Chromatium*, *Lamprocystis*, *Thiocystis*, and *Thiodictyon spp.*, as well as GSB of the genus *Chlorobium* (Bosshard *et al.*, 2000). These multiple species of PSB persist and remain active despite a recent increase in the abundance of *Chlorobium clathratiforme* (Decristophiris *et al.*, 2009; Gregersen *et al.*, 2009). In particular, '*Ca.* Thiodictyon syntrophicum' strain Cad16 accounts for over 25% of primary production and is also a prominent source of CO_2 assimilation in the dark (Storelli *et al.*, 2013).

It is unclear what factors support the limited diversity among photoautotrophs in the Mahoney Lake chemocline. Several lines of evidence indicate that of the total *Thiohalocapsa* sp. strain ML1 biomass, a relatively small proportion (~15%), is physiologically active (Overmann *et al.*, 1991, 1994). The formation of buoyant aggregates of *Thiohalocapsa* sp. causes high cell numbers to accumulate within the chemocline (Overmann & Pfennig, 1992), and the high salinity of the hypolimnion and gas vacuolation of the cells inhibit sedimentation of these aggregates (Overmann *et al.*, 1991). The elevated levels of sulfate and especially sulfide (35 mM) in Mahoney Lake may create a very narrow niche space that precludes the majority of anoxygenic photoautotrophs. Specific adaptations including elevated salt tolerance and adaptation to low light (Overmann *et al.*, 1994) may enable *Thiohalocapsa* sp. strain ML1 to persist, albeit with very slow growth rates (Overmann *et al.*, 1991, 1994).

Phylogenetic analyses of the ribosomal protein S3 indicates multiple SRB spp. are present (Fig. S8A) in the Mahoney Lake chemocline. This record of SRB is consistent with the observation that sulfate reduction provides the majority of reducing equivalents for PSB-dominated primary production, and more specifically, that some of the sulfide is supplied *in situ* rather than by vertical mixing (Overmann *et al.*, 1996a). Redox oscillations at the oxic-anoxic boundary, as is common in sediment systems (Aller, 1998), are also observed both diurnally and seasonally in Mahoney Lake. The associated small vertical excursions of the chemocline (Hall & Northcote, 1990) may help maintain a diverse population of SRBs. Alternatively, varying levels of oxygen or sulfide tolerance among taxa could result in increased diversity (Baumgartner *et al.*, 2006).

Encompassing both of these ideas, the metagenomic data presented here contain genetic evidence for both heterotrophic and autotrophic SRB, implying that fluctuations in carbon supply play a role in maintaining multiple SRB spp. in the chemocline. SRB utilize organic compounds as electron sources for cellular metabolism. However, autotrophic growth of hydrogen-utilizing SRB via the Wood-Ljungdahl pathway has been demonstrated in some species, including close relatives of some of those recovered in the present study, that is, *Desulfobacteriaceae* (Rabus *et al.*, 2013). The strong density gradient in Mahoney Lake inhibits much of the *in situ* organic matter of the chemocline from entering the layers below (Overmann *et al.*, 1991), implying it is consumed by heterotrophs within the chemocline or lost to vertical mixing and/or buoyancy. During the autumnal die-off and upwelling of PSB biomass (Overmann *et al.*, 1994), however, there is minimal concomitant change in net activity in the chemocline. This implies that the total chemocline sulfate reduction rate is relatively stable, even if the loss of this organic matter affects the fractional balance of processes (autotrophic vs. heterotrophic carbon supply). This seasonal variation in organic carbon supply may support the multiple SRB spp. observed in the Mahoney Lake chemocline.

The energetic maintenance of euxinia

Although sulfate reduction and sulfide oxidation both occur in the Mahoney Lake chemocline, such a system is not self-sustaining. To satisfy the basic principles of thermodynamics and maintain a coupled oxidative and reductive sulfur cycle, exogenous input of electrons is a stoichiometric requirement (Overmann, 1997). All of the sulfide in Mahoney Lake must be derived from sulfate reduction, with the electrons provided by respiration of organic matter. However, oxygenic primary production in the epilimnion is not sufficient to supply these electrons in the form of phytoplanktonic carbon fixed *in situ*. Instead, most of the needed organic matter must come from the surrounding landscape (Overmann *et al.*, 1996a; Overmann, 1997).

Flux balance analysis confirms this principle. From data in Overmann *et al.* (1996a), we calculate an average diffusive vertical mixing of 9.2 mmol H_2S m^{-2} day^{-1} and 23 mmol SO_4^{2-} m^{-2} day^{-1} into the chemocline (described in the SOM). The H_2S flux is only sufficient to supply ~40% of measured oxidation rates (Overmann *et al.*,

1991), implying that SO_4^{2-} must be reduced *in situ* in the chemocline to supply the remainder of the H_2S. This is consistent with suggestions that input of allochthonous – and therefore necessarily oxygenic – organic matter is required to supply the extra flux of electrons needed to sustain sulfate reduction (Overmann, 1997). The question then remains: where does all the upwelled sulfur go? The epilimnion maintains a net SO_4^{2-} deficit (and zero H_2S) relative to the hypolimnion, while sediment trap data show that <0.5% of the total upward sulfur flux sinks out of the chemocline as S^0 (Overmann *et al.*, 1994). To close mass balance, the remainder must be rafted to the shoreline in association with the annual partial die-off and buoyant dispersal of the PSB from the chemocline (Overmann *et al.*, 1994)(Fig. 10B). This relationship implies that the net sulfur cycle in the lake primarily is $SO_4^{2-} \rightarrow S^{2-}$ (H_2S) $\rightarrow S^0$, that is, the terminal sink for sulfur is significantly more reduced relative to its oxidized input (SO_4^{2-} from surrounding rocks). The ability to sustain net sulfate reduction in such a euxinic system, both in the chemocline and in the deeper hypolimnion, at first appears remarkable. However, simple free energy calculations under *in situ* conditions ($\Delta G = \Delta G^0 + RT \ln Q$) based on collated carbon and sulfur species concentrations from the literature (Northcote & Hall, 1983; Overmann *et al.*, 1991, 1996a, b) show that while Mahoney Lake represents an extreme end-member of euxinia with regard to sulfide concentrations, the H_2S:SO_4^{2-} ratio in this system still is not high enough to thermodynamically inhibit sulfate reduction (Amend & Shock, 2001; Table S10, Fig. S10). The persistence of sulfate reduction under the extreme euxinic conditions Mahoney Lake implies that – at least under oxygenated atmospheres – SRB should be active in anoxic systems throughout Earth history, regardless of the degree of euxinia.

The redox stability and stable, poised conditions of Mahoney Lake likely result from its unique combination of high salinity, high sulfur concentrations, and high allochthonous carbon loading. The more dilute conditions of Lake Cadagno – another analogue for ocean euxinia – result in greater seasonal changes in fluxes, redox variability, and microbial community succession (Bosshard *et al.*, 2000; Del Don *et al.*, 2001; Decristophiris *et al.*, 2009; Gregersen *et al.*, 2009). More generally, the concentration of free sulfide in any water column should scale directly with the extent of organic matter overloading, and the extreme case of Mahoney Lake undoubtedly is a factor in its biogeochemical and ecological stability.

Implications for biosignatures of photic zone euxinia

The chemocline of Mahoney Lake provides valuable insight into the geologic record of anoxygenic phototrophy. Because the habitat of phototrophic sulfur bacteria is restricted – requiring micro-oxic or anoxic conditions, free sulfide, and sunlight – diagenetic products of the carotenoid okenone have been invoked as markers for shallow photic zone euxinia (Brocks *et al.*, 2005; Brocks & Schaeffer, 2008). However, the synthesis of okenone is irregularly distributed among *Chromatiaceae*, both taxonomically and physiologically. Therefore, it is still debated whether diagenetic okenane in geologic sediments is of planktonic origin, or if it also may be derived from benthic mat communities (Meyer *et al.*, 2011) in which PSB are widespread and diverse (Imhoff, 2005). Mat- or benthic-derived isolates that produce okenone include *Halochromatium roseum* JA134 (Kumar *et al.*, 2007), *Thiocapsa marina* (Caumette *et al.*, 1985, 2004), and the closest relative of *Thiohalocapsa* sp. strain ML1, *Thc. halophila* (Caumette *et al.*, 1991; Kumar *et al.*, 2007).

Okenone in ML sediments (Overmann *et al.*, 1993; Coolen & Overmann, 1998) has been linked to the presence of *Thiohalocapsa* sp. strain ML1 in the water column (Overmann *et al.*, 1991). The metagenomic sequencing and phylogenetic analyses presented here show that the only detectable okenone producer in the water column is indeed this species. It is not only closely related to *Thc. halophila,* but it also appears to be more closely related to the *Trv. winogradsky* isolated from the shoreline mats (Overmann *et al.*, 1992) than to typical planktonic members of the *Chromatiaceae,* such as '*Ca.* Thiodictyon syntrophicum'. The taxonomic similarity between *Thiohalocapsa*, *Halochromatium*, and *Thiorhodovibrio* genera (Tank *et al.*, 2009; this work) suggests that a more general continuity between planktonic and benthic PSB communities exists, especially where oxygen minimum zones impinge on sediments within the photic zone. Similar to Mahoney Lake, the okenone-rich chemocline of meromictic Fayetteville Green Lake in New York hosts a diverse population of PSB, and phylogenetic analyses of clones from benthic and planktonic habitats in that lake suggest these niches host distinct PSB populations (Meyer *et al.*, 2011). Okenone-rich sediments derived from benthic mats underlying the oxic water column at Fayetteville Green Lake have challenged the current interpretation of okenone as an exclusive biomarker for planktonic PSB. In Mahoney Lake, the example of okenone production in the water column by a PSB strain that is a close relative of benthic species further highlights the difficulty in linking okenone uniquely to water column processes. While it remains assured that the recovery of okenane from ancient sediments is strong evidence for sunlit, sulfidic environments inhabited by PSB, the presence of okenane alone may not be sufficient evidence for photic zone euxinia in the open water column.

At the same time, in environments such as Mahoney Lake where planktonic PZE clearly is present, chemoautotrophic sulfide oxidation and sulfate reduction also should be suspected as co-occurring reactions in an active sulfur

cycle, mediated by a wide variety of micro-organisms. This is distinct from a cryptic sulfur cycle in which no net sulfur cycle redox processes occur (Canfield *et al.*, 2010), but rather is an expected consequence of the water column anoxia associated with high organic carbon fluxes. Under this model, both PZE and water column sulfate cycling necessarily depend on oxygenic photosynthesis to supply electrons. This implies that PZE is only possible in high productivity, low and moderate pO_2 oceans (including ocean basins with restricted circulation). Episodes of euxinia in Earth history should require a confluence of all of these conditions, perhaps helping to explain their apparent rarity (e.g., Meyer & Kump, 2008; Johnston *et al.*, 2009; Canfield, 2013; Leavitt *et al.*, 2013).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1 Supplementary methods and results.

Table S1 Metagenome summary statistics.

Table S2 Marker genes used to evaluate genome completion of the isolate genome, strain ML1, and the ML-Gamma and ML-Epsilon genomic bins.

Table S3 Marker genes used to evaluate genome completion of the Deltaproteobacteria community genome bins.

Table S4 Orthologous group markers and their abundance used to estimate the number of complete genomes in the Deltaproteobacteria community genome bins.

Table S5 Single-copy phylogenetic marker genes used for taxonomic assignment of the ML-Gamma and the ML-Epsilon genomic bins.

Table S6 List of genes from the Mahoney Lake 7-m metagenome identified and included in functional gene phylogenetic analyses.

Table S7 Number and taxonomic affiliation of key genes involved in the sulfur cycle.

Table S8 Accession numbers of the Sqr sequences included in Fig. S6.

Table S9 Summary of community genomic bins of Deltaproteobacteria spp.

Table S10 Calculations for $\Delta G = \Delta G^0 + RT\ln Q$ at 18°C for selected sulfur redox reactions in situ.

Table S11 List of organisms from which NifH/VnfH/AnfH were identified and included in the phylogenetic analysis of NifH/VnfH/AnfH identified in from the Mahoney Lake 7-m metagenome.

Fig. S1 ESOM (repeating-tiled view) bins of the tetranucleotide frequency signatures of the 7-m metagenome with bins of interest indicated.

Fig. S2 The estimated number of SRB genomes recovered from the Mahoney Lake metagenome.

Fig. S3 Maximum likelihood phylogenetic tree of 19 concatenated single-copy ribosomal proteins showing the placement of the strain ML1 and ML-Gamma compared to closely related Gammaproteobacteria spp. for which genome sequences are available.

Fig. S4 Phylogenetic diversity of RuBisCO in the Mahoney Lake chemocline. Maximum likelihood phylogenetic trees constructed from CbbL and CbbM sequences mined from the ML 7-m metagenome and the NCBI and IMG/M database.

Fig. S5 Phylogenetic diversity of the nitrogenase Fe protein in the Mahoney Lake chemocline.

Fig. S6 Maximum likelihood phylogenetic tree of translated genes sequences encoding okenone C-4/4′ ketolase (CruO), okenone accessory ketolase (CruS), carotenoid desaturase (CrtD) and methoxyneurosporine dehydrogenases (CrtD/CrtI family homologs).

Fig. S7 Maximum likelihood phylogenetic tree of Sqr. Sequences were mined from the 7-m metagenome (Table S6) and the NCBI and IMG/M database.

Fig. S8 Taxonomic diversity of SRB-affiliated SP3 proteins and the Predicted role of the ML SRB spp. in the sulfur cycle.

Fig. S9 Phylogenetic diversity of RuBisCO in the Mahoney Lake chemocline. Maximum likelihood phylogenetic trees constructed from CbbL and CbbM sequences mined from the ML 7-m metagenome and the NCBI and IMG/M database.

Fig. S10 Calculated values for $\Delta G = \Delta G^0 + RT\ln Q$ at 18 °C for selected sulfur redox reactions *in situ*, at depths above, within, and below the purple chemocline.