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Disparate distributions of chemolithotrophs containing form IA or IC large subunit genes for ribulose-1,5-bisphosphate carboxylase/oxygenase in intertidal marine and littoral lake sediments

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chemolithotroph; ribulose-1,5-bisphosphate carboxylase/oxygenase; marine; freshwater; biogeochemistry.

Abstract

The distributions of bacterial form IA and form IC ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) were investigated using Lowes Cove intertidal mudflat and Damariscotta Lake littoral sediments by PCR amplification of 492–495 bp fragments of the large subunit RuBisCO gene, *cbbL*. Genomic extracts for amplification were obtained from lake surface (upper 2 mm), mudflat surface (upper 2 mm), subsurface (5–7 cm), and soft-shell clam (*Mya arenaria*) burrow-wall sediments, as well as from a sulfide-oxidizing mat. Phylogenetic analyses of *cbbL* clone libraries revealed that Lowes Cove sediments were dominated by form IA *cbbL*-containing sequences most closely related to *cbbL* genes of sulfur-oxidizing bacteria or sulfide-oxidizing mats. In contrast, Damariscotta Lake *cbbL* clones contained primarily form IC *cbbL* sequences, which typify aerobic CO- and hydrogen-oxidizing facultative chemolithotrophs. Statistical analyses supported clear differentiation of intertidal and lake chemolithotroph communities, and provided evidence for some differentiation among intertidal communities. AMOVA and LIBSHUFF analyses of Lowes Cove libraries suggested that *M. arenaria* burrow-wall sediments did not harbour distinct communities compared with surface and subsurface sediments, but that surface and subsurface libraries displayed moderate differences. The results collectively support a conceptual model in which the relative distribution of form IA- and IC-containing bacterial chemolithotrophs depends on sulfide availability, which could reflect the role of sulfate reduction in sediment organic matter metabolism, or the presence of geothermal sulfide sources.

Introduction

Bacteria incorporate CO_2 into biomass by several mechanisms. Many aerobic chemolithotrophs use the Calvin–Benson–Bassham (CBB) cycle (Kelly & Wood, 2002). Alternatively, chemolithotrophic *Epsilonproteobacteria* use the reductive tricarboxylic acid cycle (Campbell *et al.*, 2006). Although not especially well studied, chemolithotrophic *Epsilonproteobacteria* have been recognized recently as potentially important contributors to the marine sulfur cycle (Campbell *et al.*, 2006). The 3-hydroxypropionate cycle also provides a mechanism for CO_2 fixation, although its use by chemolithotrophs thus far appears limited (Menendez *et al.*, 1999). Finally, many anaerobes incorporate CO_2 via the Wood–Ljungdhal pathway, which depends on the key enzyme carbon monoxide dehydrogenase/acetyl CoA synthase (Wood & Ljungdahl, 1991).

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) catalyzes the first reaction of the CBB cycle. It occurs in three forms that fix CO_2, and a fourth that does not (Atomi, 2002). Forms I and II occur in *Bacteria* and eukaryotic phototrophs, while form III occurs in *Archaea* (Watson *et al.*, 2000; Atomi *et al.*, 2001; Atomi, 2002; Maeda *et al.*, 2002). Among phototrophic and chemolithotrophic *Bacteria* (excluding *Epsilonproteobacteria*), form I occurs most commonly, but some chemolithotrophs also contain form II, usually together with form I (Shively *et al.*, 1998). Phylogenetic analyses of RuBisCO form I large subunit genes, *cbbL*, reveal four evolutionarily distinct clades, namely IA–ID. Of these, forms IA and IC occur most commonly in bacterial chemolithotrophs and eukaryotes; form IB occurs in some cyanobacteria and eukaryotes, and form ID appears to be limited to eukaryotes.

Numerous *cbbL* analyses have revealed that ammonia, nitrite, reduced metal and sulfur oxidizers primarily harbour form IA genes (Shively *et al.*, 1998). Many of these organisms are obligate chemolithotrophs, as they depend strictly on reduced inorganic N or S and CO_2 as energy and carbon sources, respectively (Kelly & Wood, 2002). Some form IA-containing isolates function as facultative chemolithotrophs, and grow with organic substrates or by oxidizing either sulfide or other reduced sulfur species; some grow mixotrophically with both (e.g. Gude *et al.*, 1981; Howarth *et al.*, 1999; Sorokin *et al.*, 2000; Teske *et al.*, 2000). A few notable exceptions have been observed: *Hydrogenophaga pseudoflava*, a facultative chemolithotrophic CO and hydrogen oxidizer, does not oxidize reduced sulfur species but contains form IA *cbbL* (Lee & Kim, 1998), as does *Alkalilimnicola ehrlichei* MLHE-1, an arsenite-oxidizing chemolithotroph that also oxidizes CO (Oremland *et al.*, 2002). In addition, several anoxygenic phototrophs and two nitrite oxidizers (*Nitrobacter winogradskyi* and *N. hamburgensis*) possess both form IA and form IC *cbbL* genes (Uchino & Yokota, 2003; Starkenburg *et al.*, 2006).

In contrast, form IC *cbbL* occurs in a facultatively chemolithotrophic manganese oxidizer and in many CO- and hydrogen-oxidizing facultative chemolithotrophs that preferentially use heterotrophic substrates as carbon and energy sources. A group of ammonia-oxidizing *Nitrosospira* species also contains form IC *cbbL*, the sequences of which appear monophyletic and distinct from other form IC sequences (Ütaker *et al.*, 2002; Tolli & King, 2005). All of these form IC-containing isolates differ from the majority of form IA-containing bacteria owing to their inability to grow at the expense of sulfide (Meyer & Schlegel, 1983; Friedrich & Schwartz, 1993; Caspi *et al.*, 1996; Aragno, 1998; Santini *et al.*, 2000; Francis *et al.*, 2001; Skirnisdottir *et al.*, 2001; Nanba *et al.*, 2004; Selesi *et al.*, 2005; Tolli & King, 2005).

Although chemolithotrophs play major roles in ecosystem ecology and elemental cycles (Fenchel *et al.*, 1998), their distributional patterns in aquatic systems have not been well explored. This is partly the result of the lack of diagnostic 16S rRNA gene probes. Nonetheless, use of the CBB cycle by many chemolithotrophs (e.g. Shively *et al.*, 1998; Tabita, 1999) has facilitated molecular approaches based on *cbbL*. Analyses of *cbbL* genes have revealed phylogenetically diverse chemolithotroph communities at deep-sea seeps and hydrothermal vents (Elsaied & Naganuma, 2001), as well as in BTEX (benzene-toluene-ethylbenzene-xylenes)-contaminated groundwater and chlorobenzene-contaminated aquifers (Alfreider *et al.*, 2003). In contrast, little phylogenetic diversity was observed for bacterioplankton in a redox gradient in Mono Lake, California (Giri *et al.*, 2004). Although revealing, these studies have provided only partial insights into chemolithotroph distributions, because the primers used amplified form IA (or possibly IB) *cbbL*, but apparently did not allow consideration of bacteria containing form IC genes.

We propose here that the distribution of form IA- and IC-containing chemolithotrophs in aquatic sediments reflects the relative availability of sulfide, which in turn reflects geochemical sulfide sources or the importance of sulfate as an oxidant in carbon cycling. In coastal marine sediments, sulfate reduction consumes a large fraction of organic matter, and sulfide oxidation accounts for a large fraction of oxygen uptake (Fenchel & Jørgenson, 1977; Jørgensen & Postgate, 1982; Fenchel *et al.*, 1998). This reduces the availability of organic substrates to support aerobic form IC-containing facultative chemolithotrophs while favouring the growth of sulfide-oxidizing form IA-containing chemolithotrophs (obligate and facultative). In freshwater sediments with low sulfate concentrations, a greater fraction of organic substrates and oxygen can support form IC-containing chemolithotrophs, while form IA-containing chemolithotrophs are limited by sulfide availability. This proposal is consistent with results from surveys of terrestrial systems using primers that amplify both form IA and form IC *cbbL* (Nanba *et al.* 2004; Tolli & King, 2005). In these studies, clone libraries were overwhelmingly dominated by form IC sequences, reflecting the virtual absence of sulfides (Nanba & King, 2004; Tolli & King, 2005).

We report here a test of this notion. We have examined patterns in the distributions of form IA and form IC *cbbL* in sediments of a relatively sulfide-rich intertidal mudflat and of a sulfide-poor littoral zone of a freshwater lake. DNA was extracted from surface sediments (upper 2 mm) of Damariscotta Lake, Maine, and surface, subsurface (5–7 cm), and soft-shell clam (*Mya arenaria*) burrow-wall sediments of Lowes Cove, Maine. DNA was also extracted from sulfide-oxidizing bacterial mats located on the fringe of Lowes Cove. A 492–495 bp fragment of the *cbbL* gene was amplified by PCR with primers that target both form IA and form IC *cbbL* genes (Nanba *et al.*, 2004). Phylogenetic and statistical analyses of *cbbL* clone libraries indicate that marine and freshwater sediments greatly differ in chemolithotroph community structure, with form IA dominating marine sediments and form IC dominating lake sediments. Statistical analyses of Lowes Cove surface, subsurface and *M. arenaria* burrow-wall sediments indicate varying degrees of similarity based on phylogenetic differentiation, genetic diversity and evolutionary distance measurements.

Materials and methods

Site description and sample collection

The distributions of form IA and form IC *cbbL* were examined in four distinct types of intertidal marine sediment collected from Lowes Cove, Maine, aspects of which have been described previously, including active sulfate reduction, limited oxygen penetration depths (< 2 mm)

and extensive bioturbation by a large and diverse macrobenthic community (King *et al*., 1983; King, 1986, 1990; Findlay *et al*., 1989; Sawyer & King, 1993; Hansen *et al*., 1996; Giray & King, 1997; Chung & King, 1999). Sample types comprised mats of colourless sulfide-oxidizing bacteria that occurred along the edge of the Cove in areas receiving drainage from adjacent fringing salt marshes; the upper 2 mm of surface sediment; sediment from 5–7 cm depth; and sediment from the walls of *M. arenaria* burrows.

Lowes Cove sediments used for CO, ammonium and thiosulfate oxidation assays were collected at low tide by coring with 7-cm-diameter bleach-sterilized acrylic tubes. Core contents were carefully extruded to obtain the desired depth intervals. Surface and subsurface sediments for DNA extractions were collected by coring with sterile cut-off 50-cm^3 syringes. Colourless sulfide-oxidizing bacterial mats were collected with 50-cm^3 syringes for all assays. *Mya arenaria* burrow-wall sediment was obtained by exposing burrows *in situ*, and then using a sterile spatula to scrape the inner 2 mm, which was transferred to Whirlpak bags.

Form IA and form IC *cbbL* content was also examined in freshwater sediments collected from the upper 2 mm of the littoral zone (< 1 m depth) of the mesotrophic Damariscotta Lake in Damariscotta Lake State Park (Jefferson, ME). Sediment at the collection site was composed of several centimetres of nonsulfidic sand overlying a glacial clay deposit. The water column at the sampling site was well oxygenated, but oxygen penetration depths in the sediment were < 2 mm. Sediments were collected by coring with bleach-sterilized 7-cm-diameter acrylic tubes. Sediment was collected within 2 m of the shore, and overlying water was *c*. 0.5 m deep. Cores were equilibrated briefly before removal and returned to the laboratory for processing. Overlying water was removed with a sterile syringe. Surface sediment was removed with a sterile spatula. All sediment samples were processed immediately after collection.

Oxygen microprofiles

Oxygen distributions for both Lowes Cove and Damariscotta Lake sediments were determined using 'Clark-style' oxygen microelectrodes (Revsbech, 1989) fabricated by Dr R.G. Carlton (EPRI, Inc.) with a guard cathode; tip diameters were < 5 μM and the response was stirring-insensitive. For Lowes Cove surface and mat profiles, triplicate determinations were obtained *ex situ* using intact cores exposed to air; duplicate determinations were obtained similarly for Damariscotta Lake using cores with a shallow, mixed water column (*c*. 1 cm). Triplicate Lowes Cove burrow profiles were obtained by exposing *M. arenaria* burrows in the field, plugs were transported to the laboratory for profiling, see above.

Dissolved and total reducible inorganic sulfide and dissolved sulfate

Damariscotta Lake porewater for dissolved sulfide analysis was obtained using a needle and syringe as described by King (1990) to obtain 0.5-mL volumes from the 0–0.5 and 2–2.5 cm intervals of triplicate cores. Sulfide was determined immediately after sample collection following Cline (1969). Total reducible inorganic sulfur (TRIS), consisting of elemental sulfur, solid-phase iron monosulfides and pyrite, was determined for Damariscotta Lake sediment from the 0–0.2 cm and 2–2.5 cm intervals by means of a chromium reduction method as described by Hansen *et al*. (1996). TRIS contents for Lowes Cove sediments have been previously reported by Hansen *et al*. (1996). Dissolved sulfate concentrations in Damariscotta Lake littoral-zone surface water were determined using a barium–gelatin method (King & Klug, 1982).

Carbon monoxide uptake

One gram fresh weight (gfw) of each triplicate sediment sample was transferred to 60-mL serum bottles. One millilitre of sterile artificial seawater (ASW) or filter-sterilized lake water was added to Lowes Cove and Damariscotta Lake samples, respectively. Serum bottles were sealed with gas-tight stoppers, and CO was added to the headspaces via needle and syringe to a final concentration of *c*. 200 ppm. Headspace samples were removed at intervals for 5 days by syringe and needle. Samples were analysed by gas chromatography using an RGA-3 gas chromatograph (Trace Analytical) equipped with a mercury vapour detector (see Rich & King, 1999).

Ammonium oxidation

Slurries were prepared by placing 2 gfw of each triplicate sediment sample in 50-mL sterile disposable centrifuge tubes containing 10 mL of sterile ASW (Lowes Cove samples) or deionized water (Damariscotta Lake samples) containing 1 mM ammonium chloride and 10 mM sodium chlorate to prevent nitrite oxidation (Belser & Mays, 1980). Subsamples (1 mL) were removed at intervals for up to 2 weeks, transferred to microcentrifuge tubes, and centrifuged to pellet sediment. Supernatants (800 μL) were transferred into 2-mL disposable cuvettes. Absorbance (543 nm) was determined using a Beckman DU640 spectrophotometer before and after the addition of 16 μL of 1% acidic sulfaniliamide (buffered in 10% sulfuric acid) and 16 μL of 0.1% n-(1-naphthyl-)ethylenediamine dihydrochloride (Grasshoff, 1976).

Thiosulfate oxidation

One gram fresh weight of triplicate sediment samples was added to 120-mL sterile serum bottles. Bottles were amended with 10 mL of sterile ASW or filter-sterilized lake

water containing 10 mM thiosulfate for marine and freshwater sediments, respectively, and sealed with sterile rubber stoppers. Subsamples of 300 μL were obtained at intervals for 2 days for Lowes Cove samples and for 6 days for Damariscotta Lake samples. Subsamples were transferred to 1.5-mL microcentrifuge tubes and centrifuged to pellet sediment. Supernatants were then transferred to 15-mL disposable centrifuge tubes with 2.5 mL of reaction buffer (0.1 M sodium phosphate and 1 mM EDTA, pH 8.0) and 50 μL of 4 mg mL^{-1} 5,5′-dithio-bis-(2-nitrobenzoic acid) (DNTB). DNTB reacts with thiosulfate to produce a quantifiable coloured product (Ellman, 1959). Samples were analysed on a Beckman DU640 spectrophotometer at a wavelength of 412 nm. In addition to the above protocol, two control reactions were used, including a background control with no thiosulfate addition, and autoclaved-killed controls to determine the potential for abiological thiosulfate oxidation.

DNA extraction and cbbL gene amplification

DNA was extracted from triplicate sediment or mat samples using MoBio UltraClean Soil DNA kits (MoBio Labs, Carlsbad, CA), according to the manufacturer's instructions. DNA was amplified with Master*Taq* DNA polymerase (Brinkmann Inc.) using recommended buffers, magnesium, dNTPs and Master*Taq* and primers K2f and V2r from Nanba *et al.* (2004) using an Eppendorf Mastercycler (Brinkmann Inc.) with the following conditions: initial 3-min 94 °C denaturation, 30 cycles of 94 °C for 45 s, 62 °C for 60 s and 72 °C for 60 s, and a final extension at 72 °C for 7 min. PCR products were electrophoresed in 1% agarose and visualized after staining with ethidium bromide. Products (492–495 bp) were immediately processed for cloning or stored at − 20 °C for no longer than 24 h.

Clone library construction

Triplicate PCR products from sediments and mats were pooled and purified with MoBio PCR cleanup kits (MoBio Labs, Carlsbad, CA) or Qiagen gel extraction kits. Clone libraries were constructed using an Invitrogen TOPO TA cloning kit and *Escherichia coli* TOP10 competent cells (Invitrogen Life Technologies, Carlsbad, CA). Clone colonies were arbitrarily picked and grown with shaking overnight at 37 °C in Luria-Bertani broth containing 50 μg mL^{-1} kanamycin. Cultures were centrifuged, pelleted, washed and resuspended in 10 mM Tris buffer, pH 7.8. Resuspended cells were amplified by PCR with cloning vector primers T3 and T7. PCR products of the correct size were purified with a MoBio PCR purification kit and sequenced by the University of Maine's DNA Sequencing Facility using vector primer T7, resulting in 492–495 bp of sequence. Sequences were screened by BLAST (Altschul *et al.*, 1997) to determine their similarity to published *cbbL* sequences.

Phylogenetic analyses

DNA sequences were aligned using CLUSTALX version 1.01 (Thompson *et al.*, 1997). The *cbbL* phylogeny was analysed with a neighbour-joining (NJ) algorithm with the PAUP* phylogeny analysis program (Swofford, 2003). Confidence in phylogenetic tree topology was assessed with bootstrapping by the NJ method in PAUP*.

Statistical analyses

Substrate oxidation rates were analysed with a two-tailed *t*-test ($\alpha = 0.05$). Maximum potential oxidation rates were determined by calculating slopes of linear regressions for each replicate at the maximum rate of oxidation. Significant differences in maximum potential oxidation were determined by performing a one-way ANOVA with the R statistical software package (www.r-project.org). Transformations were applied when necessary to obtain normality and constant variance. Normality was measured by means of the Lilliefors (Kolmorov–Smirnov) test, and the null hypothesis of normality was accepted at a probability level ≥ 0.15. Constant variance was measured with the Levine test, and the null hypothesis of constant variance was accepted at a probability level of ≥ 0.05. When significant differences in means were present, as measured by an *F*-statistic ($P \leq 0.05$), a Tukey HSD posthoc test was performed with 95% family-wise confidence.

Clone libraries obtained from Damariscotta Lake and Lowes Cove sediments were analysed by analysis of molecular variance (AMOVA) with ARLEQUIN (Schneider *et al.*, 2000) to estimate the significance of differences in population pairwise fixation indices (F_{ST} values) among *cbbL* libraries. ARLEQUIN was also used to estimate nucleotide diversity and average pairwise differences of aligned *cbbL* sequences. Nucleotide diversity estimates the probability that two randomly chosen homologous nucleotides will differ, while average pairwise difference estimates the number of nucleotide differences observed when each clone sequence is compared with all other clone sequences.

Sediment *cbbL* clone libraries were also analysed with the webLIBSHUFF program (http://libshuff.mib.uga.edu), which calculates homologous and heterologous coverages using a Cramer–von Mises statistic with a Monte Carlo test procedure (Singleton *et al.*, 2001). The distance matrix used in the LIBSHUFF analysis was obtained using DNADIST of the PHYLIP program [v. 3.65; J. Felsenstein; (evolution.genetics.washington.edu/phylip.htm)]. Libraries were considered significantly different at $P \leq 0.05$. A phylogenetic *P*-test was performed to test if the distribution of unique sequences

between different clone libraries displayed significant covariance with phylogeny (Martin, 2002). One thousand random trees were constructed in PAUP from combined clone libraries. The tree length of the combined library was determined by constructing a parsimony tree using the heuristic search algorithm in PAUP*. Clone libraries were considered significantly different if the actual tree length was less than the 95% lower confidence limit of the random trees. Bonferroni corrections for multiple comparisons were applied for LIBSHUFF and *P*-test analyses.

Accession numbers

Chromatium vinosum, D90204; *Synechococcus* strain CcmK, U46156; *Synechococcus* T6SY9, AY157474; *Nitrosomonas* sp. str. ENI11, AB061373; *Nitrosospira* sp. str. TCH716, AF459718; *Hydrogenophaga pseudoflava*, U55037; *Thiobacillus* sp., M34536; *Bradyrhizobium japonicum* USDA 110, AF041820; *Nitrosospira* sp. str. III2, AF426416; *Nitrosospira* sp. str. 40K1, AF426428; *Nitrosospira* sp. str. AF, AF426415; *Nitrosospira multiformis* ATCC 25196, AY157474; *Nitrobacter hamburgensis* X14 plasmid 1, CP000320; *Nitrobacter hamburgensis* X14, CP000319; *Nitrobacter winogradskyi* Nb-255, CP000115; *Ralstonia eutropha*, U20585; *Rhodobacter blasticus*, AB082959; *Solemya velum* sulfur-oxidizing symbiont, AY531637. Terrestrial clones: HM34, AY422874; PN5.81, DQ149802; PN5.58, DQ149798; GP0.95, DQ149762. Sulfide-oxidizing mat clones: CM4, AY422060; CM6R, AY422061. Hydrothermal vent clones: IA04, AY431011; Seamount clone Suiyo (IC)-3, AB181164. Manganese-oxidizing clone, L32182. Accession numbers for Lowes Cove clones: DQ683582-683664; Damariscotta Lake clones, DQ683665-683683.

Results

Oxygen distribution

Oxygen penetration depths for Lowes Cove were 1.4 ± 0.1 mm, 0.9 ± 0.2 mm and 1.2 ± 0.2 mm (means $\pm$ 1 SE) for surface, burrow and sulfide-oxidizing mat samples, respectively. Penetration depths did not differ significantly among sites based on ANOVA ($P = 0.25$). Penetration depths for burrows were more consistent than those for mats and surface sediments (Fig. 1), which contributed to the lack of statistical significance in the differences among penetration depths. Damariscotta Lake oxygen penetration depths from duplicate determinations were 0.3–0.5 mm.

Dissolved sulfide, TRIS and sulfate

Damariscotta Lake porewater sulfide concentrations for the 0–0.5 and 2–2.5 cm intervals were $\leq$ the limit of detection, about 1 μM. TRIS concentrations for 0–0.5 and 2–2.5 cm intervals were 33.6 ± 5.4 nmol gdw^{-1} and 47.0 ± 15.4 nmol gdw^{-1}, respectively. A range of about 50–140 μmol TRIS gdw^{-1} sediment has been previously reported for Lowes Cove surface, bulk and burrow sediments (Hansen *et al.*, 1996). Damariscotta Lake sulfate concentrations were 47 ± 1 μM.

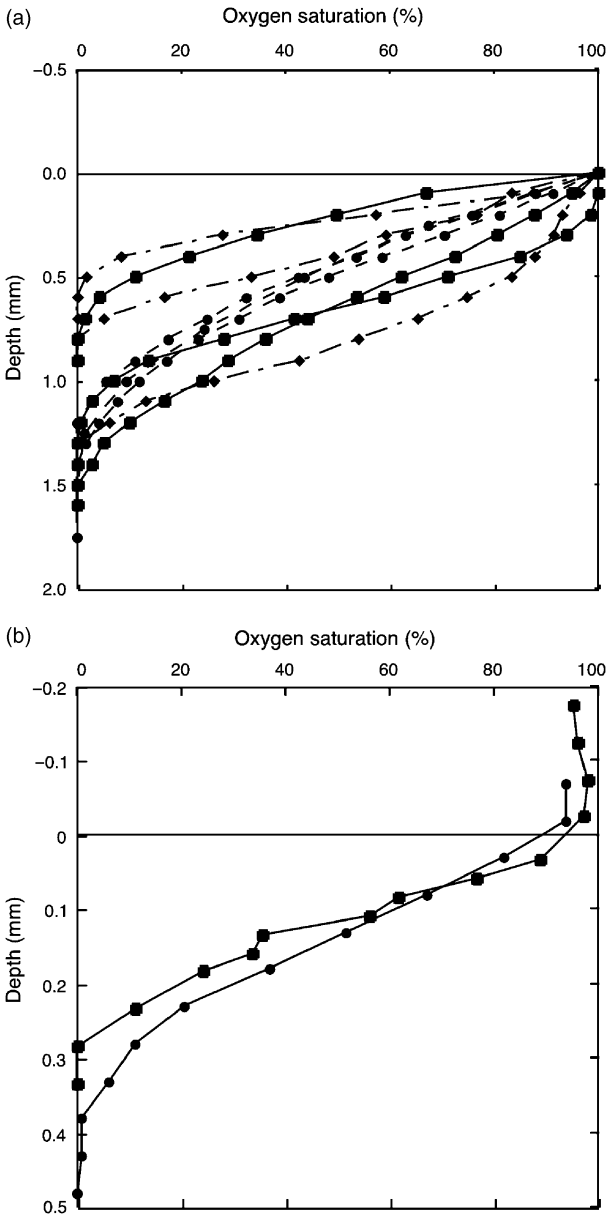

Fig. 1. A. Depth profiles of oxygen in surface (•) and burrow-wall (◆) sediments and sulfide-oxidizing mats (■) of Lowes Cove; individual symbols represent individual profiles. B. Depth profiles of oxygen in surface sediments of Damariscotta Lake; individual symbols represent individual profiles.

Substrate oxidation assays

Ammonium oxidation was detected at relatively low rates for Lowes Cove surface and burrow sediments, but not at all for Damariscotta Lake surface sediments (Table 1). Thiosulfate and carbon monoxide oxidation were readily observed in all sediments (Table 1). Transformations of both ammonium and thiosulfate data greatly improved normality and homogeneity of variances. Significant differences between site means ($P < 0.05$) were observed for ammonium and thiosulfate oxidation assays, but not for CO uptake. A Tukey HSD (95% confidence) was performed with ammonium and thiosulfate rate data. Ammonium oxidation rates in Lowes Cove surface and burrow-wall sediments were not significantly different ($P = 0.31$), but were significantly higher than in subsurface sediments ($P < 0.0008$). Thiosulfate oxidation rates for Damariscotta Lake and Lowes Cove suboxic sediments were not significantly different ($P = 0.62$). Lowes Cove surface and burrow sediments had comparable thiosulfate oxidation rates ($P = 0.78$) that were significantly higher ($P < 0.05$) than those in Damariscotta Lake and Lowes Cove subsurface sediments. All sediment types had significantly lower thiosulfate oxidation rates than Lowes Cove sulfide-oxidizing bacterial mats ($P < 0.05$).

Phylogenetic analyses

Lowes Cove sediments were dominated by form IA *cbbL*, while Damariscotta Lake sediments predominately contained form IC (Figs 2 and 3). The majority of surface (91%), subsurface (97%) and *M. arenaria* burrow-wall (86%) clones, and all clones from a sulfide-oxidizing bacterial mat were form lA. Form IA clones from Lowes Cove were phylogenetically similar to each other, although some were closely related to hydrothermal-vent clone sequences and sequences from sulfide-oxidizing mats in a noncontiguous mudflat on the Sheepscot River, Maine (see Nanba *et al.*, 2004).

Twenty-one of 23 Damariscotta Lake clone sequences (91%) were form IC. These sequences were phylogenetically diverse, and most closely related to a variety of chemolithotrophs or clone sequences reported for agroecosystem soils and volcanic deposits. The form IA clones were most closely related to *Rhodobacter blasticus*, an anyoxygenic phototroph.

Clone library statistical analyses

Nucleotide diversity and within-site average pairwise differences were similar among all libraries (Table 2). Among-site average pairwise differences for Lowes Cove microhabitats were nearly identical, while differences between Lowes Cove samples and Damariscotta Lake sediments were higher (Table 3).

Values of Wright's fixation index (F_{ST}, Table 3) indicated varying levels of genetic differentiation among clone libraries (see Hartl & Clark, 1997). The highest F_{ST} values were observed for comparisons between Lowes Cove libraries and the Damariscotta Lake sequences. Lowes Cove surface and subsurface sediment sequences showed moderate genetic differentiation, while little genetic diversity was observed among burrow-wall clones and marine surface and subsurface environments (Table 3).

LIBSHUFF analyses of clone library homologous and heterologous coverages (Table 4) indicated that Lowes Cove clone libraries were all significantly different from the Damariscotta Lake sediment library ($P = 0.001$). Among Lowes Cove microhabitats, surface and subsurface sediments contained distinct communities ($P = 0.001$), while neither of these communities was significantly different from the *M. arenaria* burrow-wall community ($P = 0.0085$).

The length of a parsimony tree containing Lowes Cove clone libraries (surface, subsurface, and *M. arenaria* burrow-wall sediments) was significantly less than the 95% lower confidence limit of 1000 random trees. *P*-tests of individual Lowes Cove libraries similarly indicated significant phylogenetic divergence of marine microhabitat populations. Removing form IC *cbbL* sequences from Lowes Cove libraries did not change the outcome of AMOVA, LIBSHUFF or *P*-test analyses.

Discussion

Although horizontal gene transfer has limited the correspondence between *cbbL* and 16S rRNA gene phylogenies (Delwiche & Palmer, 1996), results from numerous isolates indicate that the overwhelming majority of form IA-containing chemolithotrophs oxidize sulfide and other reduced sulfur species for growth (obligately or facultatively), or depend on ammonia or nitrite oxidation. In contrast, most

Table 1. Maximum potential oxidation rates for ammonium, carbon monoxide, and thiosulfate (means of triplicates ± 1 SE)

	Site				
Substrate	LC surface	LC burrow	LC subsurface	LC mat	Dscot Lake
Ammonium	15.4 ± 53.2	47.5 ± 16.9	3.4 ± 2.4	–	ND
CO	4.2 ± 7.0	4.1 ± 1.0	3.4 ± 2.4	–	6.6 ± 2.2
Thiosulfate	72.5 ± 30.8	110.0 ± 53.2	23.1 ± 2.8	363.6 ± 51.1	17.9 ± 11.1

LC, Lowes Cove; Dscot Lake, Damarsicotta Lake; ND, not detectable. Rates for ammonium oxidation in pmol gdw^{-1} h^{-1}; rates for CO uptake in nmol gdw^{-1} h^{-1}; rates for thiosulfate oxidation in μmol gdw^{-1} h^{-1}.

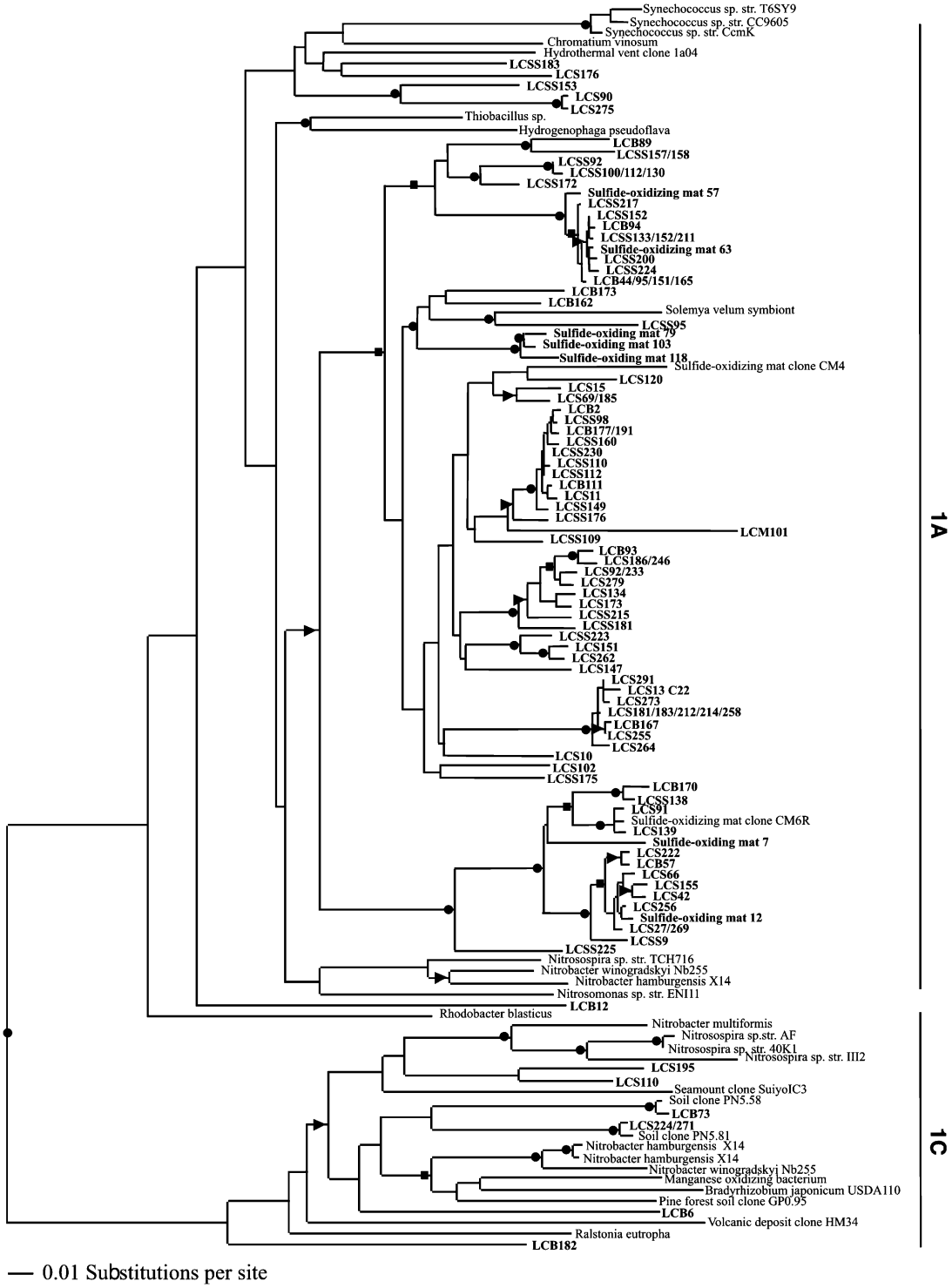

Fig. 2. Neighbour-joining tree of Lowes Cove partial *cbbL* nucleic acid sequences with a Jukes–Cantor correction. Bootstrap support (1000 replicates) is indicated as closed circles, closed squares and closed triangles for 91–100%, 81–90% and 70–80%, respectively. Lowes Cove clones shown in bold. Site abbreviations: LCS, Lowes Cove surface; LCB, Lowes Cove burrow; LCSS, Lowes Cove subsurface.

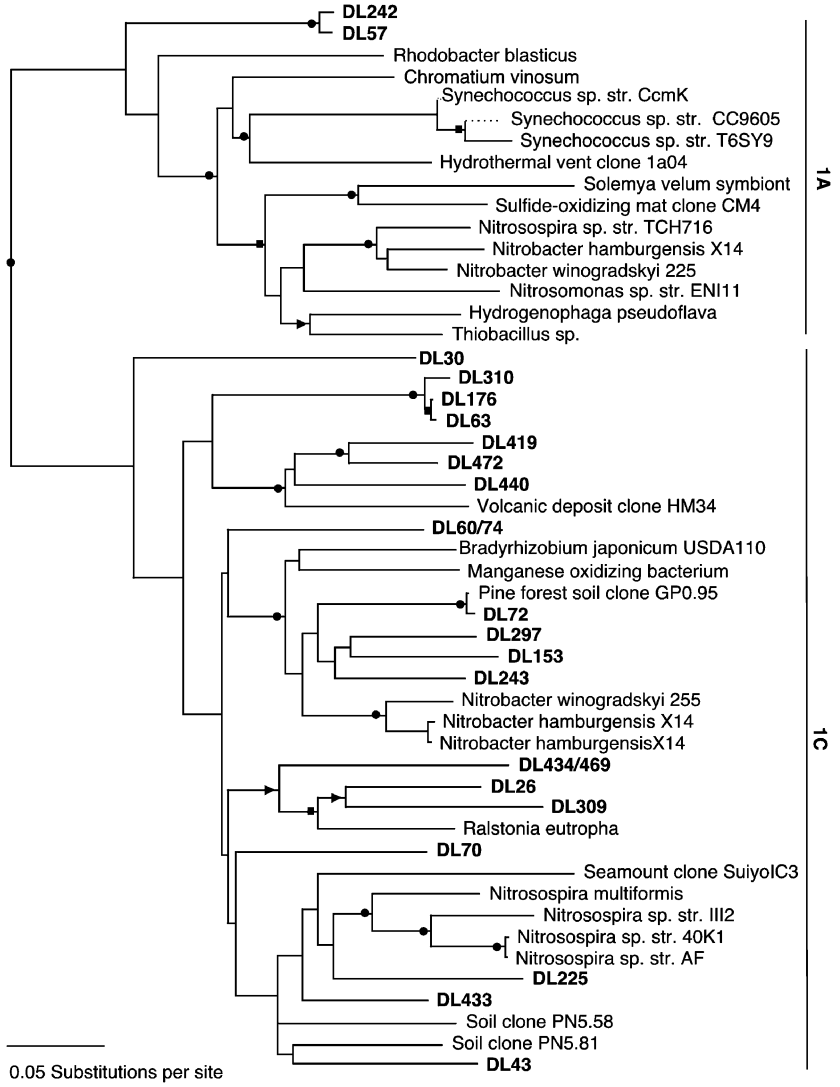

Fig. 3. Neighbour-joining tree of Damariscotta Lake partial *cbbL* nucleic acid sequences with a Jukes–Cantor correction. Bootstrap support (1000 replicates) is indicated as closed circles, closed squares and closed triangles for 91–100%, 81–90% and 70–80%, respectively.

Table 2. Nucleotide diversity (ND) and within-site average pairwise difference ($\theta[\pi]$) for Lowes Cove (LC) and Damariscotta Lake (DL) samples (means $\pm$ 1 SE)

Site	Clones	ND	$\theta[\pi]$
LC surface	45	0.22 ± 0.11	109.8 ± 48.0
LC subsurface	33	0.17 ± 0.08	85.4 ± 37.7
LC burrow	20	0.26 ± 0.13	126.1 ± 56.6
DL surface	23	0.28 ± 0.14	137.8 ± 61.3

Table 3. Among-site average pairwise differences (above diagonal) and fixation indices (F_{ST}; below diagonal)

	LCS	LCB	LCSS	DL
LCS		120.0	103.8	216.9
LCB	0.019		105.3	213.1
LCSS	0.059	0.001		222.2
DL	0.440	0.381	0.511	

All differences were significant ($P < 0.05$). LCS, Lowes Cove surface; LCB, Lowes Cove burrow; LCSS, Lowes cove subsurface (5–7 cm); DL, Damariscotta Lake.

form IC-containing chemolithotrophs assayed to date oxidize CO or H_2 (or both), but not sulfide. A relatively small group of *Nitrosospira* obligately oxidizes ammonia, but not sulfide, and contains form IC *cbbL* sequences that are phylogenetically distinct from those of CO and H_2 oxidizers. Thus, at a 'macro' level, phylogenetic distinctions among *cbbL* sequences correspond to functional distinctions.

Recently, Elsaied & Naganuma (2001) used *cbbL* primers to characterize deep-sea marine sediment and

Table 4. LIBSHUFF probability values for XY (above diagonal) and YX (below diagonal) library comparisons

Library	LCS	LCB	LCSS	DL
LCS		0.094	0.001	0.001
LCB	0.678		0.567	0.001
LCSS	0.001	0.196		0.001
DL	0.001	0.001	0.001	

Libraries were considered significantly different at 95% confidence if the XY or YX comparison had a probability value of $\leq$ 0.0085, based on a Bonferroni correction for multiple comparisons. LCS, Lowes Cove surface; LCSS, Lowes Cove subsurface (5–7 cm); LCB, Lowes Cove burrow; DL, Damariscotta Lake.

hydrothermal-vent bacterial lithotrophs. They obtained sequences that were most closely related to those of sulfide-oxidizing isolates or cyanobacteria. Alfreider *et al.* (2003) and Giri *et al.* (2004) also used *cbbL* primers to characterize contaminated groundwater sediment and bacterioplankton in Mono Lake, respectively. Alfreider *et al.* (2003) observed diverse form IA sequences, while Giri *et al.* (2004) obtained sequences most similar to an arsenite-oxidizing chemolithotroph and *Hydrogenovibrio marinus*. Selesi *et al.* (2005) used distinct *cbbL* primer sets to amplify form IA and IC *cbbL* genes from agroecosystem soils, and noted that the former were low in diversity compared with the latter. Nanba *et al.* (2004) and Tolli & King (2005) used primers that simultaneously amplify both IA and IC genes, and found form IC sequences almost exclusively in various terrestrial and agroecosystem soils.

Results from this study reveal that form IA *cbbL* dominates clone libraries obtained from genomic extracts of Lowes Cove intertidal sediments, regardless of sediment origin, i.e. surface, subsurface or burrow wall (Fig. 1). For many of the form IA clones, the closest matches based on BLAST searches include known sulfide-oxidizers or sequences from sulfide-oxidizing bacterial mats that occur in the noncontiguous Sheepscot River mudflat (Fig. 2; Nanba *et al.*, 2004). Similarities between some Lowes Cove and Sheepscot River clones suggest a possible regional-scale distribution of several closely related chemolithotrophs. However, 'rampant' horizontal transfer of RuBisCO genes, and the possibility of a high degree of sequence conservation limit inferences about the physiology and taxonomic identity of specific clones, even those that appear to be closely related (Delwiche & Palmer, 1996).

Form IC *cbbL* sequences also occur in Lowes Cove sediments (Fig. 1). These sequences are not closely related to known chemolithotrophs, including marine CO oxidizers; they do not cluster closely with *Nitrosospira* and *Nitrobacter* sequences. The latter observation may indicate that the sequences do not represent nitrifying bacteria, although this possibility cannot yet be excluded. Active aerobic CO consumption by Lowes Cove sediments (Table 1 and King, 2007) implies the presence of at least some aerobic CO-oxidizing bacteria, the overwhelming majority of which contain form IC *cbbL*. Thus, aerobic CO oxidizers may account for Lowes Cove form IC sequences and represent taxa that to date remain poorly characterized or unknown in culture.

In contrast to Lowes Cove, Damariscotta Lake surface sediment clones contain primarily form IC *cbbL* sequences (Fig. 3). Because these sequences do not appear closely related to *Nitrosospira* or *Nitrobacter* sequences, they may represent CO and hydrogen oxidizers, or perhaps iron and manganese oxidizers, as the latter also contain form IC genes (e.g. Holden & Brown, 1993; Caspi *et al.*, 1996). Several of the Damariscotta Lake form IC clones closely matched sequences from volcanic and agricultural systems (Nanba *et al.*, 2004; Tolli & King, 2005), with up to 99% identity (Fig. 3), but, as noted above for form IA clones, sequence similarity does not necessarily correlate with physiology or taxonomic identity.

Damariscotta Lake sediments also contain form IA *cbbL* sequences, although they are much less abundant than form IC sequences (Fig. 3). The Damariscotta Lake sequences are not phylogenetically related to form IA sequences obtained from Lowes Cove sediments, known sulfur oxidizers, or nitrifying bacteria. The closest match in the NCBI database for these sequences is *Rhodobacter blasticus*, an anoxygenic hydrogen- and CO-oxidizing anaerobic photolithotroph.

Based on similarities in nucleotide diversity and average pairwise differences for each library (Table 2), the chemolithotroph communities at each of the sites appear similarly diverse. More specifically, the extent of divergence or phylogenetic differentiation among the clones for a given site approximates the differentiation among clones for other sites. In contrast, average pairwise differences derived from comparisons between sites (Table 3) indicate that Lowes Cove and Damariscotta Lake clone libraries differ substantially, while clones from libraries within Lowes Cove do not (see Fig. 2).

Wright's fixation index (F_{ST}; Wright, 1951) also reveals little differentiation among Lowes Cove libraries, but significant differentiation between chemolithotrophs represented by the Lowes Cove and Damariscotta Lake libraries (Table 3). Similarly, LIBSHUFF analyses support distinct chemolithotroph communities for Damariscotta Lake and Lowes Cove, because all pairs of reciprocal comparisons between Damariscotta Lake and Lowes Cove libraries differ significantly (Table 4). LIBSHUFF analyses also indicate that Lowes Cove surface and subsurface libraries differ from each other, but that neither differs from the burrow library. In essence, surface and subsurface chemolithotroph communities contained different assemblages, most likely as a result of active burial of surface sediments by bioturbation and loss of some of the surface populations. Although *M. arenaria*

burrows represent relatively stable microhabitats that differ from surface sediments with respect to local chemical and microbiological parameters (e.g. Hansen *et al.*, 1996), they appear to integrate populations from both surface and subsurface environments.

The preponderance of form IA *cbbL* sequences closely related to sulfide oxidizers and sulfide-oxidizing mat clones in Lowes Cove but not in Damariscotta Lake is consistent with TRIS concentrations, which provide an index of sulfide availability. TRIS concentrations are about three orders of magnitude greater in Lowes Cove than in Damariscotta Lake sediments, which probably reflects a much reduced role for sulfate reduction in Damariscotta Lake carbon cycling. This can be attributed to sulfate concentrations that are about 500-fold lower for Damariscotta Lake than for Lowes Cove (47 μM vs. 25 mM). *cbbL* patterns in Lowes Cove are also consistent with maximum potential substrate oxidation patterns, because maximum potential thiosulfate oxidation rates substantially exceed rates for ammonium oxidation and CO consumption.

Substrate oxidation patterns for Damariscotta Lake are somewhat more enigmatic. While *cbbL* sequences suggest dominance of the chemolithotrophic community by CO and hydrogen oxidizers, a substantial capacity for thiosulfate oxidation was observed (Table 1), implying the possible presence of form IA-containing sulfur oxidizers, none of which are represented in the Lake clone libraries. Several nonexclusive possibilities may account for these observations. First, thiosulfate turnover in oxic freshwater sediments may involve rhodanese, a well-known thiosulfate-sulfur transferase present in lake and marine sediments (Nriagu *et al.*, 1979; Wainwright, 1980; Alexander & Volini, 1987; Kelly & Baker, 1990; Saidu, 2004) that may act independently of chemolithotrophic metabolism. Second, thiosulfate may be oxidized in freshwater sediments by bacteria that do not contain *cbbL* genes. *Silicibacter pomeroyi* provides a relevant example, because it oxidizes thiosulfate, but does not fix CO_2, contains no *cbbL* genes, and can function as a chemolithoheterotroph (Moran *et al.*, 2004). Third, some form IC-containing facultative chemolithotrophs may oxidize thiosulfate in addition to CO or hydrogen. Examples include the facultative chemolithotroph *Bradyrhizobium japonicum* USDA 110, the genome of which contains *sox* genes (Kaneko *et al.*, 2002), *Mesorhizobium thiogangeticum* (Ghosh & Roy, 2006) and *Bacillus schlegelii* (Beffa *et al.*, 1993). In the latter case, heterotrophic substrates may effectively subsidize a significant capacity for thiosulfate oxidation, even though thiosulfate may not be readily available.

Collectively, these results and results from previous analyses of agroecosystem soils and volcanic deposits support the hypothesis that the relative distribution of form IA- and form IC-containing chemolithotrophs reflects the availability of sulfide for aerobic bacterial oxidation, which in most sediments reflects the role of sulfate reduction in carbon turnover. Bulk organic matter concentrations alone cannot account for patterns of form IA and IC *cbbL* distributions within clone libraries, because both Damariscotta Lake and Lowes Cove sediments contain more organic matter than Hawaiian volcanic deposits; clone libraries from the latter are dominated by form IC sequences (Nanba *et al.* 2004) while Lowes Cove clone libraries are dominated by form IA *cbbL*.

In essence, consumption of relatively large amounts of organic matter and oxygen by sulfate reduction and sulfide oxidation, respectively, which are characteristic of coastal sediments, favours the development of form IA-containing sulfide-oxidizing chemolithotrophs, probably including obligate, facultative and mixotrophic subgroups. The relative importance of these functionally distinct subgroups remains unknown, but is a subject worthy of attention for both theoretical and practical reasons (Tittel *et al.*, 2003). In contrast, sulfate-poor systems, for example freshwater sediments and soils, appear to contain insufficient sulfide to support significant sulfide oxidizer populations, and instead favour the development of the metabolically versatile form IC-containing facultative chemolithotrophs that oxidize CO, hydrogen, organic matter, and perhaps thiosulfate to a lesser extent. Ongoing and future studies of marine and nonmarine water column systems and various sulfidic and nonsulfidic hot springs will help to clarify further the distribution of form IA- and IC-containing chemolithotrophs and their functions. If sulfide availability is a critical determinant, form IA-containing chemolithotrophs should dominate sulfidic hot springs, while form IC-containing chemolithotrophs should dominate nonsulfidic systems.

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