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Diversity and Structure of Bacterial Chemolithotrophic Communities in Pine Forest and Agroecosystem Soils†

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Obligate lithotrophs (e.g., ammonia oxidizers) and facultative lithotrophs (e.g., CO and hydrogen oxidizers) collectively comprise a phylogenetically diverse functional group that contributes significantly to carbon and nitrogen cycles in soils and plays important roles in trace gas dynamics (e.g., carbon monoxide and nitrous and nitric oxides) that affect tropospheric chemistry and radiative forcing. In spite of their diverse physiologies, facultative and obligate lithotrophs typically possess the Calvin-Benson-Bassham cycle enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisCO). In an effort designed to understand the structure of lithotrophic communities in soil, genomic DNA extracts from surface (0 to 2 cm) and subsurface (5 to 7 cm) soils have been obtained from two sites in a Georgia agroecosystem (peanut and cotton plots) and an unmanaged pine stand (>50 years old). The extracts have been used in PCR amplifications of the *cbbL* gene for the rubisCO large subunit protein. *cbbL* PCR products were cloned, sequenced, and subjected to phylogenetic and statistical analyses. Numerous novel lineages affiliated with the form IC clade (one of four form I rubisCO clades), which is typified by facultative lithotrophs, comprised lithotrophic communities from all soils. One of the form IC clone sequences clustered with a form IC clade of ammonia-oxidizing *Nitrosospira*. Distinct assemblages were obtained from each of the sites and from surface and subsurface soils. The results suggest that lithotrophic populations respond differentially to plant type and land use, perhaps forming characteristic associations. The paucity of clone sequences attributed to ammonia-oxidizing bacteria indicates that even though ammonia oxidation occurs in the various soils, the relevant populations are small compared to those of facultative lithotrophs.

Chemolithotrophic bacteria, which use inorganic compounds as electron donors for growth, have been placed into two groups based on their electron donors (for examples, see reference 34). Obligate lithotrophs include sulfide-, sulfur-, metal-, ammonium-, and nitrite-oxidizing bacteria, many of which have been described in detail. Facultative lithotrophs include aerobic hydrogen- and CO-oxidizing bacteria, of which relatively few have been described (for examples, see references 25 and 26).

Although chemolithotrophic bacteria exhibit a wide range of physiological and ecological traits, most use the Calvin-Benson-Bassham pathway to incorporate CO_2. Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisCO) plays a crucial role in this pathway (10, 11, 30, 31). rubisCO occurs in three related forms (I, II, and III) that vary in structure, catalytic properties, and substrate specificity (14, 19, 38, 39, 43, 46). Form I, which occurs in all chemolithotrophs in the bacterial domain, has been subdivided into four clades, IA to ID, based on phylogenetic analyses (28, 38, 39, 43). Form II occurs in some chemolithotrophs and phototrophs, while form III is an archaeal enzyme (14, 43). Analyses of form I rubisCO offer the greatest insights for understanding proteobacterial chemolithotroph diversity and community structure.

Sequence analyses indicate that obligate lithotrophs predominately possess form IA rubisCO, while facultative lithotrophs predominately possess form IC (38, 39, 46). Notable exceptions include some facultative sulfur oxidizers and *Hydrogenophaga pseudoflava*, a facultatively lithotrophic CO and hydrogen oxidizer in the form IA subgroup, and a number of obligately lithotrophic ammonia-oxidizing *Nitrosospira* in the form IC subgroup (45).

To date, molecular ecological studies based on rubisCO genes (e.g., the *cbbL* gene for the rubisCO large subunit protein, also known as *rbcL*) have emphasized aquatic systems and phototrophs (for examples, see references 28, 30, and 47), with relatively few analyses devoted to bacterial lithotrophs (for examples, see references 1, 4, 12, and 17) and fewer still emphasizing terrestrial systems (27, 37). A recent analysis of chemolithotrophic communities associated with volcanic deposits ranging in age from about 45 to 300 years revealed significant variations in lithotrophic populations among sites, suggesting that distinct lithotrophic communities formed in response to local edaphic factors and succession (27). The results also indicated that facultative lithotrophs dominated the communities at all sites, which was consistent with relatively rapid rates of CO and hydrogen oxidation and low to undetectable rates of ammonia oxidation (27).

Selesi et al. (37) have used two primer sets to describe the diversity of "green" (form IA) and "red" (form IC) bacterial *cbbL* genes in DNA extracts from three agricultural soils used for long-term cultivation of rye with no fertilizer, with mineral fertilizer, or with a manure addition. Their results suggested substantially greater diversity for facultative (form IC) than obligate (form IA) lithotrophs, although the relative abundance of each group was not evaluated. Their results also suggested that lithotroph diversity varied with agricultural

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treatments, particularly fertilization. Form IA clone sequences were closely associated sequences from ammonia-oxidizing bacteria, but form IC clone sequences were not linked to specific functional groups or activities.

The results presented here illustrate the diversity and structure of bacterial lithotrophic populations in surface (0 to 2 cm) and subsurface (5 to 7 cm) soils from an unmanaged pine stand and from agroecosystem soils supporting a legume (peanut, *Arachis hypogaea*) and a nonlegume (cotton, *Gossypium hirsutum*). A 489- to 495-bp fragment of the gene coding for the large subunit of the form I ribulose-1,5-bisphosphate carboxylase/oxygenase (*cbbL*) has been amplified by PCR from genomic DNA extracts from two depths at each site. Previous studies have shown that the primers amplify both form IA and IC genes (26). PCR products from the various soils have been used to construct *cbbL* clone libraries, which were compared statistically. Phylogenetic composition of libraries from each of the three sites were statistically distinct and dominated by form IC sequences. These observations indicate that soil lithotrophic communities vary with land use and crop type in agroecosystems.

MATERIALS AND METHODS

Site description, sampling, and lithotrophic activities. Soil samples were collected from a Georgia agroecosystem (planted with cotton and peanut) and a nearby, unmanaged pine stand (>50 years old) in August 2003. Various aspects of these sites have been described previously (20). Soils at both sites have been classified as plinthic paleudults and consist of a sandy loam. Organic contents were <1% for agricultural soils and >20% for pine stand soils (20). Samples for DNA extraction were collected using 7.8-cm (inner diameter) aluminum core tubes. Intact cores were sectioned, and subsamples from 0- to 2-cm and 5- to 7-cm depth intervals (below the O horizon, where present) were transferred to sterile Whirlpak sample bags and then frozen at −20°C until processed further. The litter layer was removed from pine stand soils prior to sectioning.

Triplicate soil samples from each depth and site were used to assess potential ammonium oxidation rates following a previously described method (for examples, see reference 16). Briefly, 3-gram fresh weight (gfw) samples were transferred to 50-cm^3 disposable centrifuge tubes. Twenty milliliters of deionized water containing 1 mM ammonium chloride and 10 mM sodium chlorate (a nitrite oxidation inhibitor) were added to each tube. The tubes were capped and incubated horizontally at ambient laboratory temperature with shaking (100 rpm). At intervals, the tube contents were subsampled and analyzed for nitrite content using a colorimetric method after centrifugation (16). Nitrite production rates were considered equivalent to ammonium oxidation rates.

Maximum potential CO oxidation rates were measured in parallel by transferring triplicate 5-gfw samples from each depth and site to 110-cm^3 jars that were subsequently sealed with neoprene stoppers. CO was added to the jar headspaces to create approximately 200-ppm initial concentrations. CO uptake was monitored by sampling jar headspaces at intervals and measuring CO concentrations by gas chromatography (20).

DNA extraction and PCR amplification of *cbbL*. DNA was extracted from triplicate 10-g (fresh weight) soils from 0- to 2-cm depth and 5- to 7-cm depth at each site, using a bead-beating method with an Ultraclean Mega Soil DNA extraction kit (MoBio Labs, Inc., Carlsbad, CA). A 489- to 495-bp fragment of *cbbL* was amplified using primers K2f and V2r, which were modified from those used by Xu and Tabita (47) and which target motifs TT(I)KPKLG and V(A)V GKLEG, respectively (27). Primer sequences were as follows: K2f, 5′-ACC A[C/T]CAAGCC[G/C]AAGCT[C/G]GG-3′; V2f, 5′-GCCTTC[G/C]AGCTTGC C[G/C]ACC[G/A]C-3′. PCR amplification was performed as described previously (26). The presence and size of PCR products were determined by electrophoresis in 1% agarose with ethidium bromide staining. PCR products were used within a few hours or stored at −20°C overnight for cloning.

***cbbL* clone libraries and sequence analysis.** Triplicate PCR products from each site were combined and cloned into *Escherichia coli* using TOPO TA cloning kits for sequencing according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). Transformed colonies were arbitrarily selected from each library and grown overnight in 1 ml Luria-Bertani broth. Clone screening was performed by centrifuging each turbid suspension, and rinsing the pellet once in Tris-EDTA. Clones were screened for the presence of inserts by PCR amplification using vector-specific primers (T3 and T7) according to the manufacturer's directions. PCR products were sequenced by the University of Maine Sequencing Facility using vector primer T3 and an ABI model 377 sequencer (Applied Biosytems, Foster City, CA). Clone sequences have been deposited in GenBank with accession numbers DQ149730 to DQ149802.

Clone sequences were subjected to BLAST screening (2) to determine their similarity to known *cbbL* sequences. Sequences for which *cbbL* was the most similar match were translated (http://bio.lundberg.gu.se/edu/translat.html) to obtain the inferred amino acid sequences. The correct reading frames were determined from the presence of diagnostic motifs, including forward and reverse primer sites. Inferred amino acid sequences were aligned with corresponding *cbbL* sequences from known lithotrophic bacteria with ClustalX software, version 1.83 (44); manual adjustments were made as necessary. Phylogenetic analyses were performed with PAUP, version 4.0b10 (Sinauer Associates, Inc., Sunderland, MA), using a neighbor-joining algorithm. LIBSHUFF (40) was used to estimate homologous and heterologous coverage of clone libraries as a function of evolutionary distance for pairwise reciprocal comparisons (library A compared with library B and vice versa). The significance of differences in coverage versus evolutionary distance between libraries was estimated by a bootstrap algorithm. Differences in coverage were considered significant at P values of ≤ 0.05. Analysis of molecular variance (AMOVA), as implemented with Arlequin (35), was used to estimate the significance of differences in population pairwise fixation indices (F_{ST} values) among soil libraries. Arlequin was also used to estimate nucleotide diversity, average pairwise differences, and mismatch distributions for each library. For a given set of aligned sequences within a library, nucleotide diversity estimates the probability that two randomly chosen homologous nucleotides will differ; average pairwise difference estimates the number of nucleotide differences observed when each clone sequence is compared with all other clone sequences.

Nucleotide sequence accession number. Clone sequences have been deposited in GenBank with accession numbers DQ149730 to DQ149802.

RESULTS

Nitrite accumulated linearly over time (not shown) for 7 days during assays of maximum potential ammonium oxidation rates in cotton and peanut soils. Rates varied between 0.4 to 0.6 nmol N oxidized g $soil^{-1}$ h^{-1}, with no significant variation among sites ($P > 0.1$). In contrast, nitrite did not accumulate in pine stand soils; thus, ammonium oxidation was undetectable.

Maximum potential CO oxidation rates likewise varied little among cultivated soils. CO was consumed by soils from surface and subsurface cotton and peanut soils with rates ranging from 1.6 to 1.9 nmol CO oxidized g $soil^{-1}$ h^{-1}. None of the rates for cultivated soils differed significantly. Cultivated soil rates were considerably and significantly ($P < 0.05$) less than rates for pine stand soils. The latter ranged from 13 to 83 nmol CO oxidized g $soil^{-1}$ h^{-1}; in addition, pine stand surface rates significantly ($P < 0.05$) exceeded subsurface rates.

Totals of 45, 95, and 77 clone sequences were obtained from cotton, peanut, and pine stand soil clone libraries, respectively (Table 1). Typically, clone yields were greater for subsurface soils; this was especially pronounced for the cotton soils, from which only 4 clones were obtained from the 0- to 2-cm depth in spite of repeated attempts to amplify and clone PCR products. Overall, the clone sequences yielded 31, 16, and 27 unique sequences for the cotton, peanut, and pine soil libraries, respectively. Each unique sequence was considered a haplotype or operational taxonomic unit due to the absence of data that clearly links the level of *cbbL* differentiation with distinct operational taxonomic units.

Nucleotide diversity and average pairwise difference values were greater in cotton and pine subsurface soil libraries ($P < 0.05$) than in surface soil libraries. In contrast, diversity indices were similar for surface and subsurface peanut soil libraries

TABLE 1. Total number of clones sequenced and number of haplotypes analyzed in Arlequin

Site[a]	No. of clones (no. of haplotypes)	VP[b]	ND[c]	θ[π][c]
CT 0–2	4 (1)	0	0.00 (0.00)	0.00
CT 5–7	41 (30)	286	0.23 (0.11)	112.0 (54.5)
PN 0–2	40 (2)	94	0.10 (0.05)	48.8 (24.0)
PN 5–7	55 (14)	119	0.07 (0.04)	36.4 (17.8)
GP 0–2	14 (4)	32	0.04 (0.02)	17.6 (9.3)
GP 5–7	63 (23)	240	0.15 (0.07)	72.9 (35.3)
CT (all)	45 (31)	291	0.29 (0.11)	112.6 (54.7)
PN (all)	95 (16)	157	0.14 (0.07)	66.9 (32.3)
GP (all)	77 (27)	240	0.13 (0.065)	66.2 (32.0)
0–2 cm (all)	58 (7)	148	0.13 (0.07)	65.6 (31.8)
5–7 cm (all)	159 (67)	306	0.19 (0.09)	93.4 (44.6)

[a] PN, peanut; CT, cotton; GP, pine.
[b] Number of variable sites (out of 489 to 495) in each library. VP, variable positions.
[c] Nucleotide diversity (ND) and average pairwise difference (θ[π]) are expressed as means (±1 standard deviation) for each site.

TABLE 2. Corrected average pairwise differences and F_{ST}

Site[b]	Result[a] for site:										
	CT 2	CT 5	GP 2	GP 5	PN 2	PN 5	CT	PN	GP	2	5
CT 2		63.60	89.23	67.02	84.52	91.59					
CT 5	0.33		48.56	24.72	26.84	13.91					
GP 2	0.86	0.34		10.99	48.57	82.19					
GP 5	0.46	0.22	0.13		24.13	55.82					
PN 2	0.64	0.25	0.54	0.27		51.27					
PN 5	0.72	0.17	0.71	0.50	0.55						
CT								7.6	25.9		
PN							0.09		32.8		
GP							0.24	0.33			
2											14.4
5										0.14	

[a] Corrected average pairwise differences (θ[π]) are shown above diagonal and F_{ST} values are shown below the diagonal. All values are significant at a P value of ≤0.05.
[b] Abbreviations: PN, peanut; CT, cotton; GP, Georgia pine. CT, PN, and GP refer to pooled libraries for each site; 2 and 5 refer to pooled libraries for the 0- to 2-cm and 5- to 7-cm depths from each site, respectively.

($P > 0.05$) (Table 1). The general trend for increased diversity with depth (significant at $P < 0.05$) was apparent from a comparison of all sequences from 0 to 2 cm with all sequences from 5 to 7 cm (Table 1); differences between surface and subsurface diversity indices were statistically significant ($P < 0.05$). The fixation index (F_{ST}) derived from AMOVA analysis, 0.142, also supported a significant albeit moderate sequence differentiation between surface and subsurface soils (Table 2).

A comparison of pooled sequences from each site (surface plus subsurface) revealed that the peanut and pine soil *cbbL* libraries were similarly diverse ($P < 0.05$) (Table 1). Both libraries were significantly less diverse ($P < 0.05$) than the cotton library. Differences between cotton, pine, and peanut *cbbL* libraries were not statistically significant ($P > 0.1$), however, when two form IA cotton clone sequences were removed from the comparisons (not shown).

AMOVA-derived F_{ST} values revealed significant phylogenetic sequence differentiation between cotton and pine and between peanut and pine clone library sequences but suggested that peanut and cotton sequences overlapped to a greater extent (Table 2). The degree of differentiation among sites was apparent from phylogenetic analyses that showed that pine sequence clusters were largely distinct from those of peanut and cotton, which overlapped (Fig. 1).

For all sites and depths, the majority of *cbbL* sequences clustered with the form IC clade; only two sequences were identified as form IA *cbbL* (Fig. 1). Few of the clone sequences clustered with *cbbL* sequences from known lithotrophs. Two clone sequences from cotton soils were most similar to a sequence from *Bradyrhizobium japonicum* USDA 6, while a third cotton soil sequence was most similar to sequences from two *Burkholderia* strains. One of the form IC cotton clone sequences (CT5-13) (Fig. 1) clustered with sequences from a *Nitrosospira* clade, but the two form IA clone sequences (CT5-6 and CT5-121) were not closely associated with known ammonia oxidizers or other taxa (Fig. 1). Indeed, the form 1A clone sequence, CT5-121, was divergent from other form IA sequences.

The phylogenetic differentiation among clone sequences from the cultivated and pine stand sites was supported by results from mismatch and LIBSHUFF analyses. The former revealed distinctly different patterns for each of the libraries, with cotton the most divergent (Fig. 2). LIBSHUFF analyses suggested that all three sites were significantly differentiated ($P < 0.01$ for all comparisons; not shown), with or without inclusion of the form IA cotton sequences.

DISCUSSION

In contrast to phytoplankton communities (for examples, see references 28 and 30), the structure, diversity, and dynamics of bacterial lithotrophs have been poorly studied. Only a few studies have used molecular approaches to address ecological questions about bacterial lithotrophs, and most of those have emphasized populations associated with elevated sulfide (for examples, see references 12, 16, and 32). Analyses of lithotrophic communities in soil have been even more limited, with only two reports based on use of *cbbL* as a PCR target (27, 37).

Nanba et al. (27) amplified a 492- to 495-bp region of *cbbL* genes from genomic extracts of volcanic deposits and from sulfide-oxidizing mats using primer sequences employed here. Their results and subsequent studies have indicated that these primers, modified from Xu and Tabita (47), amplified a phylogenetically broad range of prokaryotic *cbbL* genes, encompassing phototrophs and sulfide, methane, ammonium, hydrogen, and CO oxidizers (C. Weber, L. Nigro, and G. King, unpublished data). Nanba et al. (27) observed distinct lithotrophic assemblages associated with volcanic deposits at different stages of succession. These assemblages were comprised exclusively of form IC *cbbL* sequences representative of facultative lithotrophs. The molecular results were consistent with microbial activity assays, which did not detect ammonium oxidation.

Selesi et al. (37) have designed primers to independently amplify *cbbL* form IA and IC genes. They assessed *cbbL* diversity in genomic extracts from rye-planted soils that were subjected to three fertilization treatments. In general, form IA diversity was substantially less than that of form IC, and fertilized soils supported more diverse *cbbL* sequences than nonfertilized soils (37). In addition, most of the clone sequences

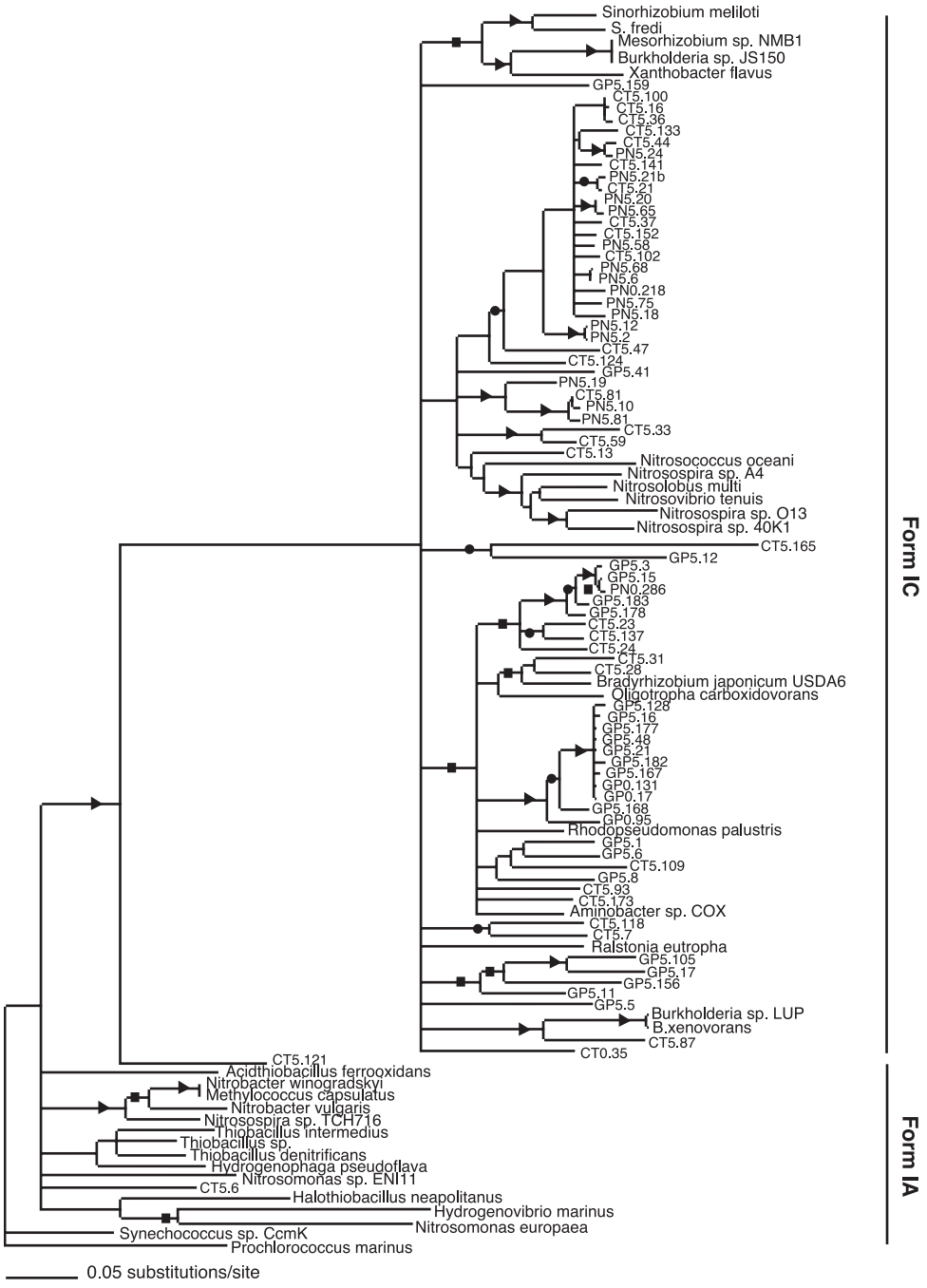

FIG. 1. Phylogram from neighbor-joining analysis of aligned nucleotide sequences for bacterial form I *cbbL*, with bootstrap support (1,000 replicates) indicated at the nodes: ●, 70 to 79%; ■, 80 to 89%; ▲, 90 to 100%. CT, PN, and GP refer to cotton-, peanut-, and pine stand-derived clones, respectively. *Synechococcus* sp. CcmK was used as an out-group to root the tree for visual representation. GenBank accession numbers for sequences from cultures are as follows: *Aminobacter* sp. strain COX, AY422046; *B. japonicum* USDA6, AY422048; *Burkholderia* sp. strain JS150, AY422049; *Burkholderia* sp. strain LUP, AY422050; *Hydrogenophaga pseudoflava*, U55037; *Hydrogenovibrio marinus*, D43622; *Mesorhizobium* sp. strain NMB1, AY422051; *Methylococcus capsulatus*, AF447860; *Nitrosomonas europaea*, AF426427; *Nitrobacter vulgaris*, L22885; *Nitrobacter winogradskyi* strain IFO14297, AF109915; *Nitrosomonas* sp. strain ENI-11, AB061373; *Nitrosospira* sp. strain O13, AF426422; *Nitrosospira* sp. strain 40K1, AF426428; *Nitrosospira* sp. strain III2, AF426416; *Nitrosospira* sp. strain AF, AF426415; *Nitrosospira* sp. strain TCH716, AF459718; *Oligotropha carboxidovorans*, Y422052; *Prochlorococcus marinus*, AE017162; *Ralstonia eutropha*, U20585; *Sinorhizobium meliloti*, AF211846; *Sinorhizobium fredii* USDA 205, AY422053; *Stappia aggregata*, AY422055; *Synechococcus* sp. strain CcmK, U46156; *Thiobacillus denitrificans*, L42940; *Thiobacillus ferrooxidans*, X70355; *Thiobacillus intermedius* K12, AF046933; *Thiobacillus neapolitanus*, AF038430; *Thiobacillus* sp. strain M34536; *Xanthobacter* sp. strain COX, AY422057.

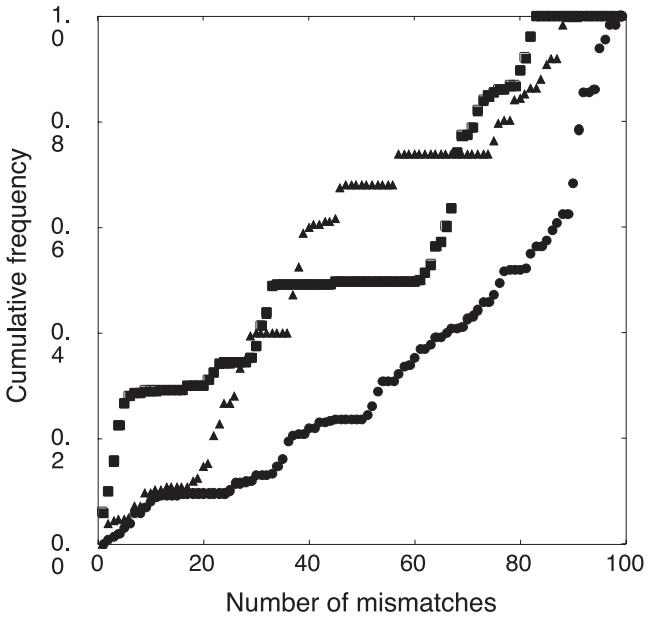

FIG. 2. Cumulative mismatch for combined cotton-, peanut-, and pine stand-derived clone libraries. The cumulative mismatch frequency is plotted versus mismatch, which is the number of base position differences obtained in pairwise comparisons of a given sequence in a library with all other aligned sequences in that library. The frequency of any given level of mismatch is estimated from the number of occurrences of that level in all sequence comparisons divided by the total number of occurrences for all levels of mismatch.

were not associated with sequences from known taxa, which suggested that the soils likely harbor numerous novel facultative lithotrophs, for which functions were not described.

The results presented here provide the first comparisons of bacterial *cbbL* diversity in agroecosystem and forest soils, surface and subsurface soils, and in soils supporting legume (peanut) and nonlegume (cotton) crops. The data support several trends. First, as in previous studies (27, 37), most of the *cbbL* clone sequences do not cluster closely with sequences from known lithotrophs (Fig. 1). While this lack of correspondence reflects in part the limited attention given to lithotrophs, it also suggests that many novel soil lithotrophs remain to be isolated and, perhaps, identified in existing cultures.

At present, the ecological roles of novel soil lithotrophs appear largely unknown. Three clone sequences, CT5-28, CT5-31, and CT5-87, clustered with *cbbL* sequences from nitrogen-fixing, CO-oxidizing heterotrophs, e.g., *Oligotropha carboxidovorans*, *Bradyrhizobium japonicum*, and *Burkholderia xenovorans*, which suggests possible functional traits for the organisms from which the clones were derived, although such inferences are limited by extensive horizontal gene transfer for rubisCO genes (6). The remaining clone sequences may also have been derived from CO or hydrogen oxidizers, since bacteria in this group typically contain form IC *cbbL* (21) and since CO oxidation was readily measurable for all sites (see above and reference 20). Further, plant roots, especially those of legumes (e.g., peanut), produce CO or CO and hydrogen (22, 23, 36). Several reports have documented elevated numbers and activities of hydrogen oxidizers in legume rhizosphere soils (5, 8, 36). Such in situ "enrichments" likely affect lithotroph community structure and diversity.

The potential significance of CO oxidizers as a source of novel facultative lithotroph *cbbL* sequences is illustrated by recovery of numerous novel CO dehydrogenase large subunit gene sequences from the agroecosystem soils used in this study (S. Cleave et al., unpublished data; G. King and K. Dunfield, unpublished data) and from similar results of a study of volcanic deposits (10). These observations reflect the presence of a very diverse facultatively lithotrophic community, the extent of which has been previously unappreciated. Since facultative lithotrophs contribute to organic matter mineralization and include numerous nitrogen-fixing bacteria (20), this group merits considerably more attention.

In addition to sequences from facultative lithotrophs, at least two, and as many as six, cotton library sequences may have been derived from obligate lithotrophs. Clones CT5-6 and CT5-121 cluster with form 1A sequences; they also contain a glycine codon characteristic of obligate lithotrophs (27). Neither sequence, however, is closely associated with sequences from known lithotrophs, so the function(s) of the parent bacteria cannot be inferred with certainty. A third cotton library sequence, CT5-13, clusters at the base of a distinct and coherent *Nitrosospira* clade containing form IC *cbbL* (Fig. 1) and may represent an ammonium oxidizer. This clade includes both marine and terrestrial representatives, but additional effort is needed to determine its phylogenetic breadth.

The presence and relative abundance in peanut and cotton clone libraries of *cbbL* sequences that might have been derived from ammonium oxidizers can be anticipated from assays of maximum potential ammonium oxidation rates. The observed activities for peanut and cotton soils are low compared to reports of ammonium oxidation in other cultivated soils (for an example, see reference 29), and no activity was detected in pine soils. The measured rates are also four- to fivefold lower than maximum potential CO oxidation rates for the same soils expressed on a mole per weight basis. Thus, it is not surprising that putative ammonia oxidizer sequences account for only 6% or less of the unique clones observed in cultivated soil libraries, while no such sequences were detected in pine soil libraries.

The sequence results support a second general trend. Specifically, statistical analyses show that clone sequences are not randomly distributed among the soils but constitute distinct assemblages as a function of sample depth and land use. Similar results have been obtained from numerous studies of bulk microbial diversity in soils (for examples, see references 3, 7, 9, 13, 15, 18, 33, and 41). F_{ST}, the fixation index, which is derived from AMOVA, measures genetic diversity within a population compared to total diversity among populations (24, 35); a zero value indicates that populations are highly similar, while a value of one indicates that they are entirely different. Moderate and statistically significant differentiation in composition exists between depths for all sites (Table 1) and among the three sites when using pooled libraries (Table 2). The greatest differentiation in this study occurs between pine and agroecosystem sites, and the least differentiation occurs between cotton and peanut soils (Tables 1 and 2). Differentiation among sites is also strongly supported by different mismatch frequency patterns (Fig. 2) and by results of pairwise comparisons of clone libraries using LIBSHUFF. These comparisons

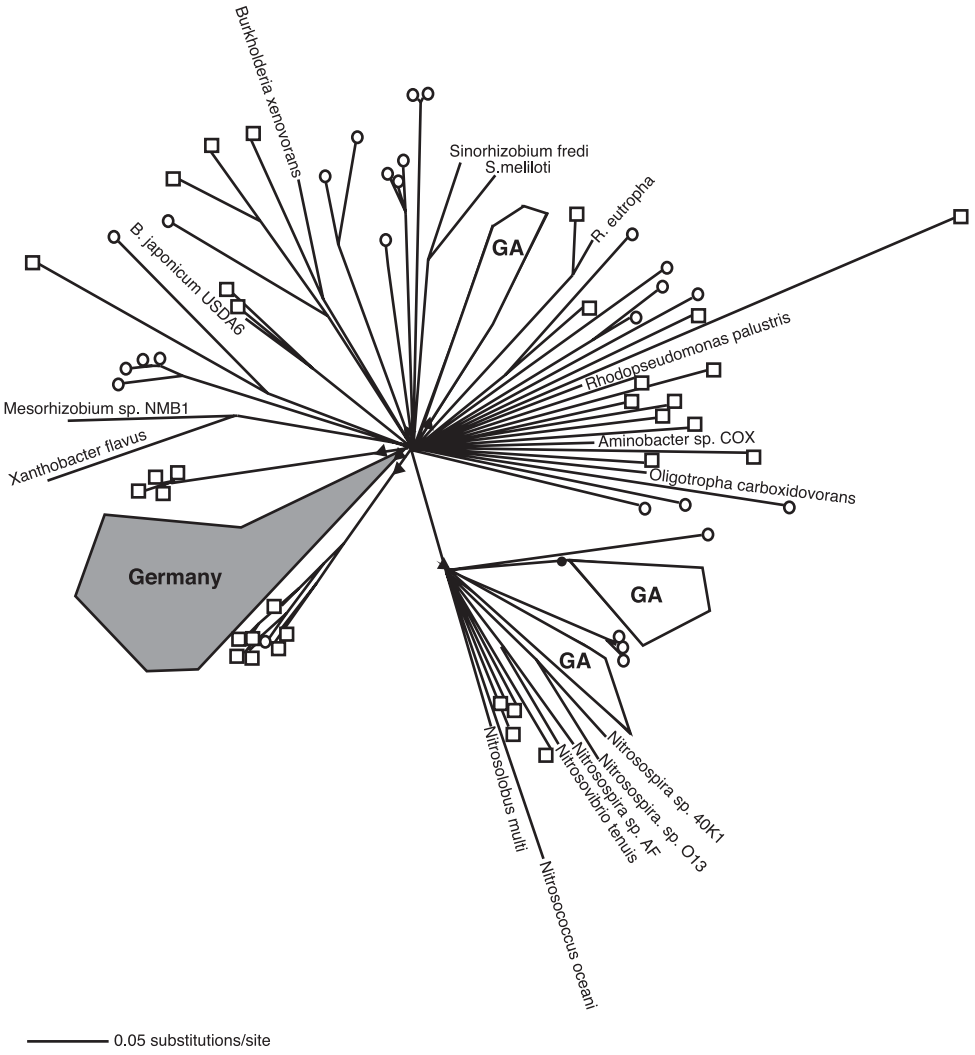

FIG. 3. Unrooted phylogram from neighbor-joining analysis of aligned nucleotide sequences for bacterial form IC *cbbL* sequences from cotton, peanut, and pine stand libraries (open squares and polygons) and from libraries of Selesi et al. (37) (closed circles and gray polygons; accession numbers AY572110 to AY572155), with bootstrap support (1,000 replicates) indicated at the nodes: ●, 70 to 79%; ■, 80 to 89%; ▲, 90 to 100%.

are all highly significant, indicating that the composition of each of the libraries is distinct. The results collectively suggest that lithotrophic community structure likely responds to the impacts of land use on variables such as moisture, temperature, and organic concentrations and that different dominant populations are selected in response to changes in these variables.

While lithotroph community structure varies among sites, diversity of each of the libraries varies less so (Table 1). When comparing diversity indices calculated using all sequences for each library, the cotton library is significantly more diverse than the peanut and pine libraries, which are equally diverse. This suggests that the diversity of a specific lithotrophic community may respond to variations in plant populations, perhaps through interactions in the rhizosphere, or in the case of crop plants, to differences in cultivation. No statistically significant differences exist among sites, however, when comparing diversity indices calculated using only the form 1C sequences (i.e., excluding 2 form 1A cotton sequences). This indicates that the diversity of obligate lithotrophs, such as ammonia oxidizers, may be more sensitive than the diversity of facultative lithotrophs to among-site variations in physical, chemical, and biological variables and that the magnitude of total lithotroph diversity for a given soil depends more on the presence of obligate than facultative lithotrophs.

Library diversity and composition varied with depth for cotton and pine but not peanut soils. In particular, cotton and pine subsurface clone library diversity significantly exceeds that of surface soils. This is evident from estimates of nucleotide diversity and average pairwise differences (Table 1). Increased subsurface diversity is also apparent from numbers of unique clone sequences for each soil (Fig. 1). Six- and sevenfold-more unique sequences occur in clone libraries from subsurface than surface soils for pine and cotton sites, respectively. Differences with depth for the pine soils likely reflect physical differentiation at the site into distinct litter, "O" and "A" horizons (20). The presence of an "O" horizon immediately above the 0- to

2-cm-depth interval soils of the "A" horizon may alter organic availability and favor a less diverse lithotroph community than at greater depths. Controls of diversity as a function of depth for cotton and peanut soils are less obvious but may involve variations in physical variables, such as temperature and water content, and biological variables, such as root distribution and root CO and hydrogen production (22, 23). All of these variables change in response to tillage and are known to affect the distributions and activities of microbial populations (for an example, see reference 42).

In addition to apparent differences in community structure at local scales (e.g., surface versus subsurface) and mesoscales (pine stand versus cultivated soils), structural differences are also evident at larger scales. A comparative analysis indicates that German and Georgian soils harbor divergent populations of lithotrophs containing form IC *cbbL* genes (Fig. 3). Sequences from both soil systems are distributed across a comparably broad phylogenetic range but cluster in distinct groups. Differences in soil type, cultivation practices, crop types, and temperature regimens, among other variables, undoubtedly account for differences in structure, even though the lithotrophs at both sites may be functionally similar.

Collectively, results from a previous analysis of volcanic deposits (27), the comparison between Georgian and German soils, and other results presented here document a remarkable diversity among terrestrial facultative lithotrophs, specific populations of which vary substantially in their distribution across a range of local to continental scales. Assemblages of these populations and their abundance relative to obligate lithotrophs may reflect a number of variables, including the availability of organic and reduced inorganic substrates. Results from this and previous studies indicate that the biogeography and dynamics of this phylogenetically very diverse group of bacteria can be analyzed using molecular approaches based on rubisCO genes in lieu of 16S rRNA genes, which are unlikely to prove adequate.

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REFERENCES

1. **Alfreider, A., C. Vogt, D. Hoffman, and W. Babel.** 2003. Diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from groundwater and aquifer microorganisms. Microb. Ecol. **45:**317–328.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935–1943.
4. **Campbell, B. J., and S. C. Craig.** 2004. Abundance of reverse tricarboxylic acid cycle genes in free-living microorganisms at deep-sea hydrothermal vents. Appl. Environ. Microbiol. **70:**6282–6289.
5. **Cunningham, S. D., Y. Kapulnik, and D. A. Phillips.** 1986. Distribution of hydrogen metabolizing bacteria in alfalfa soils. Appl. Environ. Microbiol. **52:**1091–1095.
6. **Delwiche, C. F., and J. D. Palmer.** 1996. Rampant horizontal transfer and duplication of rubisCO genes in eubacteria and plastids. Mol. Biol. Evol. **13:**873–882.
7. **Dilly, O., J. Bloem, A. Vos, and J. C. Munch.** 2004. Bacterial diversity in agricultural soils during litter decomposition. Appl. Environ. Microbiol. **70:** 468–474.
8. **Dong, Z., and D. B. Layzell.** 2001. H_2 oxidation, O_2 uptake and CO_2 fixation in hydrogen treated soils. Plant Soil **229:**1–12.
9. **Drijber, R. A., J. W. Doran, A. M. Parkhurst, and D. J. Lyon.** 2000. Changes in soil microbial community structure with tillage under long-term wheat-fallow management. Soil Biol. Biochem. **32:**1419–1430.
10. **Dunfield, K. E., and G. M. King.** 2004. Molecular analysis of carbon monoxide-oxidizing bacteria associated with recent Hawaiian volcanic deposits. Appl. Environ. Microbiol. **70:**4242–4248.
11. **Ellis, R. J.** 1979. The most abundant protein in the world. Trends Biochem. Sci. **4:**241–244.
12. **Elsaied, H., and T. Naganuma.** 2001. Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. Appl. Environ. Microbiol. **67:**1751–1765.
13. **Feng, Y., A. C. Motta, D. W. Reeves, C. H. Burmester, E. van Santen, and J. A. Osborne.** 2003. Soil microbial communities under conventional-till and no-till continuous cotton systems. Soil Biol. Biochem. **35:**1693–1703.
14. **Finn, M. W., and F. R. Tabita.** 2003. Synthesis of catalytically active form III ribulose-1,5-bisphosphate carboxylase/oxygenase in Archaea. J. Bacteriol. **185:**3049–3059.
15. **Furlong, M. A., D. R. Singleton, D. C. Coleman, and W. B. Whitman.** 2002. Molecular and culture-based analysis of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. Appl. Environ. Microbiol. **68:**1265–1279.
16. **Giray, C., and G. M. King.** 1997. Effect of naturally-occurring bromophenols on sulfate reduction and ammonia oxidation in sediments from a Maine mudflat. Aquat. Microb. Ecol. **13:**295–301.
17. **Giri, B. J., N. Bano, and J. T. Hollibaugh.** 2004. Distribution of RubisCO genotypes along a redox gradient in Mono Lake, California. Appl. Environ. Microbiol. **70:**3443–3448.
18. **Ibekwe, A. M., A. C. Kennedy, P. S. Frohne, S. K. Papiernik, C.-H. Yang, and D. E. Crowley.** 2002. Microbial diversity along a transect of agronomic zones. FEMS Microbiol. Ecol. **39:**183–191.
19. **Jordan, D. B., and W. L. Ogren.** 1981. Species variation in the specificity of ribulose bisphosphate carboxylase/oxygenase. Nature **291:**513–515.
20. **King, G. M.** 2000. Impacts of land use on atmospheric carbon monoxide consumption by soils. Glob. Biogeochem. Cycles **14:**1161–1172.
21. **King, G. M.** 2003. Molecular and culture-based analysis of aerobic carbon monoxide oxidizer diversity. Appl. Environ. Microbiol. **69:**7257–7265.
22. **King, G. M., and H. Crosby.** 2002. Impacts of plant roots on soil CO cycling and soil-atmosphere CO exchange. Glob. Change Biol. **8:**1085–1093.
23. **King, G. M., and M. Hungria.** 2002. Soil-atmosphere CO exchanges and microbial biogeochemistry of CO transformations in a Brazilian agroecosystem. Appl. Environ. Microbiol. **68:**4480–4485.
24. **Martin, A. P.** 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. Appl. Environ. Microbiol. **68:**3673–3682.
25. **Meyer, O., K. Frunzke, D. Gadkari, S. Jacobitz, I. Hugendieck, and M. Kraut.** 1990. Utilization of carbon monoxide by aerobes: recent advances. FEMS Microbiol. Rev. **87:**253–260.
26. **Morsdorf, G., K. Frunzke, D. Gadkari, and O. Meyer.** 1992. Microbial growth on carbon monoxide. Biodegredation **3:**61–82.
27. **Nanba, K., G. M. King, and K. Dunfield.** 2004. Analysis of facultative lithotrophic distribution and diversity on volcanic deposits by use of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Appl. Environ. Microbiol. **70:**2245–2253.
28. **Paul, J., A. Alfreider, and B. Wawrik.** 2000. Micro- and macrodiversity in *rbc*L sequences in ambient phytoplankton populations from the southeastern Gulf of Mexico. Mar. Ecol. Prog. Ser. **198:**9–18.
29. **Phillips, C. J., D. Harris, S. L. Dollhopf, K. L. Gross, J. I. Prosser, and E. A. Paul.** 2000. Effects of agronomic treatments on structure and function of ammonia-oxidizing communities Appl. Environ. Microbiol. **66:** 5410–5418.
30. **Pichard, S. L., L. Campbell, and J. H. Paul.** 1997. Diversity of the ribulose bisphosphate carboxylase/oxygenase form I gene (*rbcL*) in natural phytoplankton communities. Appl. Environ. Microbiol. **63:**3600–3606.
31. **Raven, J. A.** 1996. Inorganic carbon assimilation by marine biota. J. Exp. Mar. Biol. Ecol. **203:**39–47.
32. **Robinson, J. R., J. L. Stein, and C. M. Cavanaugh.** 1998. Cloning and sequencing of a form II ribulose-1,5-bisphosphate carboxylase/oxygenase from the bacterial symbiont of the hydrothermal vent tubeworm *Riftia pachyptila*. J. Bacteriol. **180:**1596–1599.
33. **Salles, J. F., J. A. van Veen, and J. D. van Elsas.** 2004. Multivariate analyses of *Burkholderia* species in soil: effect of crop and land use history. Appl. Environ. Microbiol. **70:**4012–4020.
34. **Schlegel, H. G., and H. W. Jannasch.** 1981. Prokaryotes and their habitats, p. 41–82. *In* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes: a handbook on habitats, isolation, and identification of bacteria. Springer-Verlag, New York, N.Y.
35. **Schneider, S., D. Roesseli, and L. Excoffier.** 2000. Arlequin version 2.000, a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.
36. **Schuler, S., and R. Conrad.** 1991. Hydrogen oxidation in soil following

rhizobial H_2 production by a *Vicia faba-Rhizobium leguminosarum* symbiosis. Biol. Fertil. Soils **11:**190–195.
37. **Selesi, D., M. Schmid, and A. Hartmann.** 2005. Diversity of green-like and red-like ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes (*cbbL*) in differently managed agricultural soils. Appl. Environ. Microbiol. **71:**175–184.
38. **Shively, J. M., W. Devore, L. Stratford, L. Porter, L. Medlin, and S. E. Stevens, Jr.** 1986. Molecular evolution of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisCO). FEMS Microbiol. Lett. **37:** 251–257.
39. **Shively, J. M., G. van Keulen, and W. G. Meijer.** 1998. Something from almost nothing: carbon dioxide fixation in chemoautotrophs. Annu. Rev. Microbiol. **52:**191–230.
40. **Singleton, D. R., and M. A. Furlong.** 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl. Environ. Microbiol. **67:**4374–4376.
41. **Smit, E., P. Leeflang, S. Gommans, J. van den Broek, S. van Mil, and K. Wernars.** 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl. Environ. Microbiol. **67:**2284–2291.
42. **Stearman, G. K., and J. E. Matocha.** 1992. Soil microbial population and nitrogen-transforming bacteria from 11-year sorghum tillage plots. Soil Tillage Res. **22:**299–310.
43. **Tabita, F. R.** 1999. Microbial ribulose-1,5-bisphosphate carboxylase/oxygenase: a different perspective. Photosynth. Rev. **60:**1–28.
44. **Thompson, J. D., T. J. Gibson, F. Jeanmougin, and D. G. Higgins.** 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **24:**4876–4882.
45. **Utåker, J. B., K. Anderson, Å. Aakra, B. Moen, and I. F. Nes.** 2002. Phylogeny and functional expression of ribulose 1,5-bisphosphate carboxylase/oxygenase from the autotrophic ammonium-oxidizing bacterium *Nitrospira* sp. isolate 40KI. J. Bacteriol. **184:**468–478.
46. **Watson, G. M., and F. R. Tabita.** 1997. Microbial ribulose-1,5-bisphosphate carboxylase/oxygenase: a molecule for phylogenetic and enzymological investigation. FEMS Microbiol. Lett. **146:**13–22.
47. **Xu, H. H., and F. R. Tabita.** 1996. Ribulose-1,5-bisphosphate carboxylase/oxygenase gene expression and diversity of Lake Erie planktonic microorganisms. Appl. Environ. Microbiol. **62:**1913–1921.