

Significant Role for Microbial Autotrophy in the Sequestration of Soil Carbon

Hongzhao Yuan,^{a,b} Tida Ge,^a Caiyan Chen,^a Anthony G. O'Donnell,^c and Jinshui Wu^a

Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Hunan, China^a; Graduate University of Chinese Academy of Sciences, Beijing, China^b; and Institute of Agriculture, Faculty of Natural and Agricultural Sciences, University of Western Australia, Crawley, Western Australia, Australia^c

Soils were incubated for 80 days in a continuously labeled $^{14}\text{CO}_2$ atmosphere to measure the amount of labeled C incorporated into the microbial biomass. Microbial assimilation of ^{14}C differed between soils and accounted for 0.12% to 0.59% of soil organic carbon (SOC). Assuming a terrestrial area of $1.4 \times 10^8 \text{ km}^2$, this represents a potential global sequestration of 0.6 to 4.9 Pg C year⁻¹. Estimated global C sequestration rates suggest a “missing sink” for carbon of between 2 and 3 Pg C year⁻¹. To determine whether $^{14}\text{CO}_2$ incorporation was mediated by autotrophic microorganisms, the diversity and abundance of CO_2 -fixing bacteria and algae were investigated using clone library sequencing, terminal restriction fragment length polymorphism (T-RFLP), and quantitative PCR (qPCR) of the ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) gene (*cbbL*). Phylogenetic analysis showed that the dominant *cbbL*-containing bacteria were *Azospirillum lipoferum*, *Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, *Ralstonia eutropha*, and *cbbL*-containing chromophytic algae of the genera *Xanthophyta* and *Bacillariophyta*. Multivariate analyses of T-RFLP profiles revealed significant differences in *cbbL*-containing microbial communities between soils. Differences in *cbbL* gene diversity were shown to be correlated with differences in SOC content. Bacterial and algal *cbbL* gene abundances were between 10^6 and 10^8 and 10^3 to 10^5 copies g⁻¹ soil, respectively. Bacterial *cbbL* abundance was shown to be positively correlated with RubisCO activity ($r = 0.853$; $P < 0.05$), and both *cbbL* abundance and RubisCO activity were significantly related to the synthesis rates of [^{14}C]SOC ($r = 0.967$ and 0.946 , respectively; $P < 0.01$). These data offer new insights into the importance of microbial autotrophy in terrestrial C cycling.

Global warming in the last 100 years is believed to be closely associated with increases in the concentration of atmospheric CO_2 , due primarily to the increased use of fossil fuels since the industrial revolution (26). Terrestrial ecosystems have been recognized as major sinks for global CO_2 emissions and are of significant interest because of their potential to mitigate atmospheric CO_2 (17). It has been estimated that the soil C pool is about twice as large as the atmospheric pool (6). The Calvin-Benson-Bassham cycle is the major and most widely distributed pathway for CO_2 fixation. In terrestrial ecosystems, the Calvin cycle is found in diverse organisms, from bacteria and algae to green plants (35). Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) catalyzes the first, rate-limiting step in the Calvin cycle that enables 10^2 Pg of inorganic atmospheric CO_2 to be converted into organic cellular constituents every year (34). RubisCO is central to primary productivity on land and in the oceans and has been well characterized in aquatic environments and in plant and pure cultured microorganisms (15). However, microbial autotrophs can be difficult to culture, and relatively few studies have reported on RubisCO activities in soils.

RubisCO is found in four forms (I, II, III, and IV) with each form having a different structure, catalytic activity, and O_2 sensitivity (35). Form I RubisCOs are composed of four large subunit dimers containing catalytically active amino acid residues, as well as eight additional small subunits. Form II RubisCOs exist only in certain photosynthetic bacteria and in peridinin-containing dinoflagellates. In addition to forms I and II, there are two groups of structurally related proteins that have been designated form III and form IV RubisCOs that consist only of large subunits and lack several of the key active-site residues necessary for the carboxylation of ribulose 1,5-bisphosphate (RuBP) (10). Of the different

forms, form I RubisCOs are thought to predominate in soils and are found in plants, algae, cyanobacteria, and autotrophic bacteria (38). The key active-site residue in the large subunit of form I RubisCO is encoded by the *cbbL* gene (16). Phylogenetic studies on the *cbbL* gene sequences have shown that *cbbL* genes encoding form I RubisCO can be further assigned to one of four clades, IA to ID (27, 35). These clades encompass the obligate autotrophic bacteria, including some marine cyanobacteria (*Synechococcus* and *Prochlorococcus*) (form IA RubisCO), the facultative autotrophic bacteria (form IC), green plants, green algae, and some cyanobacterial sequences (form IB), and the chromophytic algae (form ID 35). As such, RubisCO form I-encoding *cbbL* genes have been used as functional markers for molecular ecological studies of CO_2 assimilative autotrophs in aquatic systems (27). However, the genetic diversity of CO_2 fixation in soil microbial autotrophs has not been studied in any detail (32, 33).

Autotrophic microorganisms and macroalgae are known to contribute significantly to CO_2 assimilation in aquatic systems such as the oceans and wetlands (31) but have not generally been thought to have a key role in CO_2 fixation and sequestration in soils. This is despite the fact that microbial autotrophs have been reported in a number of soil studies. O'Donnell et al. (24), work-

Received 15 September 2011 Accepted 13 January 2012

Published ahead of print 27 January 2012

Address correspondence to Jinshui Wu, jswu@isa.ac.cn.

H.Y. and T.G. contributed equally to this study.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.06881-11

TABLE 1 Characteristics of the paddy and upland soils used in this study

Soil ^a	Site/position	Soil type	pH	CEC (mol kg ⁻¹)	Clay content (%)	SOC (g kg ⁻¹)	Total N (g kg ⁻¹)
P1	113°11'E, 28°8'N	Fluvisol	5.2	6.2	25.0	15.9	1.56
P2	113°11'E, 28°8'N	Fluvisol	4.7	7.2	11.3	14.8	1.64
P3	111°31'E, 29°13'N	Ultisol	5.1	12.2	11.5	17.9	1.74
U1	113°3'E, 28°12'N	Fluvisol	6.0	11.9	34.1	16.9	1.88
U2	113°5'E, 28°12'N	Ultisol	4.6	10.3	41.6	9.07	1.19
U3	111°31'E, 29°13'N	Ultisol	5.2	11.5	43.9	5.63	0.76

^a P1, P2, and P3 were paddy soils, and U1, U2, and U3 were upland soils.

ing with soil and compost isolates, have shown that certain actinomycetes, predominantly *Streptomyces*, can grow on carbon monoxide as their sole carbon source by first oxidizing CO to CO₂ and then fixing CO₂ using RubisCO and phosphoribulose kinase (2). Other studies have shown that microbial autotrophy is evident in many taxa, including aerobic, CO-oxidizing bacteria such as *Bradyrhizobium japonicum*, *Sinorhizobium meliloti*, and purple sulfide-oxidizing bacteria such as *Chromatium vinosum* (23, 32, 40).

Thus, while organisms with the genetic potential to fix CO₂ are widespread in soils, their capacity to fix CO₂ and their importance in terrestrial C cycling have yet to be fully investigated. In this paper, *cbbL* gene diversity and RubisCO activity were investigated in six different soils where ¹⁴C₂ incubation studies had shown significant incorporation of ¹⁴C into the soil microbial biomass. The significance of the work in terms of global carbon cycling and the potential for microbial autotrophy is discussed.

MATERIALS AND METHODS

Soil preparation. Three paddy soils (P1, P2, P3) and three upland soils (U1, U2, and U3) were sampled from the subtropical region of China. The sampling sites of the soils had a mean annual temperature of approximately 16.8°C and an annual rainfall of about 1,400 mm. For each soil, a bulk sample was collected from the plough layer (depth, 0 to 20 cm), hand-sorted to remove visible plant residues, and then sieved by passage through a 5-mm mesh. Soil pH was determined at a soil-to-H₂O ratio of 1:2.5 (wt/vol) using a pH meter (Delta 320, Mettler-Toledo Instruments Ltd., China), and clay content was determined using the pipette method (22). Soil organic C (SOC) content and total N were measured by dry combustion using an elemental analyzer (Vario MAX C/N, Elementar, Germany), and cation exchange capacity (CEC) was measured by titration (28). Site information and soil properties were as shown in Table 1. Prior to use, the paddy soils P1, P2, and P3 were flooded with distilled water while the other soils were adjusted to 45% of field water holding capacity. To accommodate changes in microbial activity following disturbance (3), all soils were left to equilibrate prior to analysis for 10 days after flooding or rewetting.

Incubation with ¹⁴C-labeled CO₂. For each soil, two sets of microcosms with four replicates each were prepared by weighing 1.0 kg fresh soil (in an oven-dried form) into plastic containers (10 cm in diameter and 22 cm in height). One set was used as the control and was covered with a sheet of 0.7-cm-thick dark plastic foam which blocked light but allowed aeration. Both sets of microcosms were transferred to the growth chamber (area, 80 by 250 cm; height, 120 cm; 29 April 2009, China patent no. ZL2006100197402) and incubated for 80 days in a continuously ¹⁴C₂-labeled atmosphere generated from NaH¹⁴CO₃ solution (1.0 M at a radioactivity of 1.68 × 10⁴ Bq μg⁻¹ C). The incubation system also provided artificial light (approximately 500 mmol photons m⁻² s⁻¹ parabolic aluminum reflector [PAR]) between 8:00 a.m. and 8:00 p.m., maintained temperatures of 22°C between 8:00 p.m. and 8:00 a.m. and 32°C between 8:00 a.m. and 8:00 p.m., and held relative humidity at 80 to 90% throughout the incubation period. At the end of the 80-day incubation, soils were removed from the microcosms, mixed thoroughly, and then divided into

three separate portions. One portion was immersed immediately in liquid nitrogen, freeze-dried, and stored in 10-ml sterile centrifuge tubes at -70°C for future DNA extraction, *cbbL* amplification, and RubisCO activity analyses. Another was air-dried, ground to pass through a 100-mesh filter, and used for [¹⁴C]SOC determination (43). The remaining portion was used immediately for determination of ¹⁴C-labeled microbial biomass carbon ([¹⁴C]MBC) (41). The synthesis rates (RS) of [¹⁴C]SOC (g C m⁻² day⁻¹) were calculated using the formula

$$RS = {}^{14}\text{C} - \text{SOC} \times \{1/[3.14 \times (D/2)^2]\}/T \quad (1)$$

where *D* represents the internal diameter (m) of the container and *T* represents the incubation time (80 days), respectively.

Microbial DNA extraction. Microbial DNA was extracted in triplicate from 0.5 g freeze-dried soil using the SDS-GITC-PEG method described by Chen et al. (5). The extracted pellet was dissolved in 50 μl sterilized H₂O and the DNA concentration determined using a spectrophotometer (Nanodrop; PeqLab, Germany). Prior to real-time quantitative PCR (qPCR), the DNA solution was purified using a DNA purification kit (Tiangen, China) and diluted to a final concentration of approximately 5 ng μl⁻¹ DNA using sterilized H₂O to provide the soil DNA template.

PCR amplification and terminal restriction fragment length polymorphism (T-RFLP) determination of *cbbL* genes. Bacterial *cbbL* genes (IA and IC) and chromophytic algal *cbbL* genes (ID) were separately amplified by PCR using the primers described by Nanba et al. (23) and Paul et al. (27), using forward primers labeled at the 5' end with 6-carboxy-fluorescein (FAM) (Invitrogen, China).

Briefly, each individual PCR mixture (in triplicate) contained approximately 50 ng soil DNA, 100 pmol of the primers, 200 μM deoxynucleoside triphosphates (dNTPs) (Tiangen, China), 1 U *Taq* polymerase (Tiangen, China) in 1× reaction buffer provided with the enzyme (Tiangen, China), made up to 50 μl with sterilized H₂O. Amplification was done in a Mastercycler PCR machine (model 5333; Eppendorf, Germany) using programs of 3 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 62°C, and 1.5 min at 72°C and ending with a 10-min extension at 72°C (bacterial *cbbL* gene) and 3 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 52°C, and 1.5 min at 72°C and finally with a 10-min extension at 72°C (chromophytic algal *cbbL* gene).

Labeled PCR products were purified using an agarose gel DNA purification kit (Tiangen, China). Portions (10 μl) of the purified solution (approximately 30 ng μl⁻¹) were separately digested with enzyme HhaI (for bacterial *cbbL* gene fragments) and RsaI (for the chromophytic algal *cbbL* gene fragments; TaKaRa, Japan). Digestion products were then analyzed using an automated sequencer (model 373A; Applied Biosystems, Weiterstadt, Germany) by the Sunny Company, China.

Data comprising 48 T-RFLP profiles (24 soils incubated in light with two gene targets) were processed using the Gene Scan analysis software package (version 2.1; Applied Biosystems). The relative abundance (Ap) of each T-RF was calculated from $Ap = n_i/N \times 100$, where *n_i* represents the peak height of one distinct T-RF and *N* is the sum of all peak heights in the profile. Minor peaks, where the relative abundance was <1%, were regarded as background noise (19) and not used in the statistical analysis. T-RFs greater than 10% of the total peak height were regarded as dominant fragments. Canonical correspondence analysis (CCA) of the T-RFLP

profiles was done in CANOCO 4.5 for Windows (Microcomputer Power, Ithaca, NY) (39) to assess the relationship between microbial community structure and soil physiochemical parameters SOC, total N (TN), CEC, pH, and C:N ratio.

Cloning and sequencing of *cbbL* genes. The same primer sets that were used for the T-RFLP analysis, without the fluorescent labels, were used to separately amplify the bacterial and chromophytic algal *cbbL* genes in the DNA templates of soils P1 and U1 (the representatives for paddy and upland soil, respectively). PCR products were purified and cloned into *Escherichia coli* DH5 α using the pGEM-T easy vector system (Promega, Mannheim, Germany), followed by "blue-white screening." Randomly selected clones were then screened for positive inserts by PCR using the SP6 and T7 primers (29) and sequenced by BGI (Wuhan, China). The sequences of *cbbL* clones were checked for close relatives and taxonomic assignment to known *cbbL* sequences using BLAST (1). Sequence identity of >97% was defined as an operational taxonomic unit (OTU). Bacterial and chromophytic algal *cbbL* clone sequences were each aligned using Clustal X 1.83, and two neighbor-joining trees were produced from the alignments using MEGA 4.0 (37). Bootstrap analysis of 1,000 replicates was used to estimate the stability of tree topologies.

Quantification of *cbbL* genes. Bacterial and chromophytic algal *cbbL* genes in the DNA template (1 μ l) of all of the soils were analyzed separately by real-time qPCR (21) using the primers described above. For each of the microbial types, triplicate reactions were prepared in 384-well PCR microplates (Axygen) containing 1 \times SYBR Premix ExTaq (Takara Bio Inc., Shiga, Japan), 0.15 μ M each primer (Invitrogen, China), 1 \times ROX (provided with SYBR Premix ExTaq), and 1 μ l soil DNA template (5 ng μ l⁻¹) and then amplified in an ABI Prism 9700 real-time PCR system (PerkinElmer, Applied Biosystems) using a thermal protocol for both bacterial and chromophytic algal *cbbL* genes: 30 s at 95°C followed by 40 cycles of 10 s at 95°C, 40 s at 60°C, and 30 s at 72°C. A standard curve ranging from 10² to 10⁸ bacterial or chromophytic algal *cbbL* copies μ l⁻¹ was generated using 10-fold serial dilutions of plasmids linearized by Sall (TaKaRa, Japan) with 10¹⁰ copies μ l⁻¹ partial bacterial or chromophytic *cbbL* sequence from environmental samples. A set of the reaction mixtures for the standard curve (1 μ l) was also prepared and carried out in parallel with that for the soil *cbbL* genes. The *cbbL* copies in the reaction mixtures of soils were automatically analyzed using the SDS 2.3 software provided with the real-time PCR system. The efficiency of real-time qPCRs was 95% for bacterial *cbbL* and 96% for algal *cbbL* gene (based on the slope of the standard curve).

RubisCO enzyme activity analysis. Total soil protein was extracted after 80 days of soil incubation using the method described by Takai et al. (36). Freeze-dried soil samples (2 g, four replicates) were placed in 10-ml centrifuge tubes and suspended in 6 ml protein extract containing 100 mM Tris-HCl (pH 7.8) and 1 mM dithiothreitol (DTT) (Sigma). The soil suspension was thoroughly disrupted by ultrasonication (JY92-II Scientz, China) in an ice bath and centrifuged at 20,000 \times g for 20 min at 4°C. The supernatant was amended with solid ammonium sulfate to reach 80% saturation, stirred for 30 min, and then centrifuged at 4°C (20,000 \times g, 20 min). The resultant pellets were dissolved in 50 μ l extract and used for determining RubisCO activity.

RubisCO activity in the protein extracts was measured in a reaction mixture (1.5 ml) containing the buffer system, substrates, cofactor, and coupling enzymes prepared as described by Takai et al. (36). After incubation at 30°C in a water bath for 10 min to recover enzyme activity, the absorbance of reaction mix (E_o) was determined at 340 nm using a spectrophotometer (UV-2450; Shimadzu, Japan). Following the addition of substrate (50 μ l 25 mM ribulose biphosphate), the absorbance (E_t) was determined at a reaction time of 30 s. RubisCO activity (nmol CO₂ g⁻¹ soil min⁻¹) was calculated using the formula

$$\text{RubisCO activity} = \frac{(E_o - E_t)V}{2\epsilon dtw} \quad (2)$$

where V represents the volume of the reaction mixture (ml); ϵ , the absor-

TABLE 2 Amounts of [¹⁴C]SOC and [¹⁴C]MBC and synthesis rates of [¹⁴C]SOC in six cropland soils

Soil ^a	Amt (mg kg ⁻¹) ^b		Synthesis rates of [¹⁴ C]SOC (g C m ⁻² day ⁻¹)
	[¹⁴ C]SOC	[¹⁴ C]MBC	
P1	46.41 \pm 3.56 B	4.52 \pm 0.27 C	0.074 \pm 0.003
P2	64.61 \pm 2.65 A	10.36 \pm 1.46 A	0.103 \pm 0.002
P3	60.99 \pm 7.33 A	7.61 \pm 0.42 B	0.097 \pm 0.006
U1	21.94 \pm 3.36 C	3.74 \pm 0.38 C	0.035 \pm 0.003
U2	26.36 \pm 2.08 C	4.75 \pm 0.57 C	0.042 \pm 0.002
U3	8.44 \pm 1.52 D	1.55 \pm 0.23 D	0.013 \pm 0.001

^a Soils are described in Table 1.

^b Means followed by the same letter are not significantly different ($P > 0.05$) between the different soils.

bency (6.22 \times 10⁻³ ml nmol⁻¹ cm⁻¹); d , the optical path length of the cuvette (cm); t , the reaction time (min); and w , the weight of soil (g).

Statistical analysis. Data were processed using Excel 2000 for the means and the standard errors. Multiple comparisons of significant differences were made using one-way analysis of variance (ANOVA) followed by a Duncan test ($P < 0.05$). Correlation analyses were done using the Pearson correlation method with significance defined at the 0.05 level unless otherwise stated. All analyses were performed using SPSS 13.0 software for Windows XP.

Nucleotide sequence accession numbers. Clones determined in the present study have been deposited in GenBank under accession numbers HQ174564 to HQ174665 for the bacterial *cbbL* gene and HQ184440 to HQ184456 for the chromophytic algal *cbbL* gene.

RESULTS

Amount of ¹⁴CO₂ assimilation in different soils during incubation. After 80 days of incubation, the amounts of ¹⁴CO₂ incorporated into the soil organic carbon and microbial mass were determined. Radioactivity in soils incubated in the dark was barely detectable, while significant amounts of [¹⁴C]SOC and [¹⁴C]MBC were recovered from soils incubated in the light (Table 2). The amounts of [¹⁴C]SOC and [¹⁴C]MBC ranged from 8.44 (U3) to 64.61 (P3) mg kg⁻¹ and from 1.55 (U3) to 10.36 (P2) mg kg⁻¹, respectively. The soils differed significantly in terms of incorporation of SOC and MBC (Table 2). The amounts of [¹⁴C]SOC and [¹⁴C]MBC incorporated were highly correlated ($r = 0.945$; $P < 0.05$) and were generally higher in paddy than in upland soil ($P < 0.05$). Correlation analysis also showed that the synthesis rates of [¹⁴C]SOC were closely related to RubisCO activities and the abundance of *cbbL* genes in the soils ($r = 0.946$ and 0.967 , respectively; $P < 0.01$).

Phylogenetic analysis of *cbbL* gene clones from soils. A bacterial *cbbL* gene phylogenetic tree was constructed from the 38 bacterial *cbbL* sequences (OTUs) of the test soils and 16 selected, published bacterial *cbbL* reference sequences from GenBank. The bacterial *cbbL* gene sequences were recovered in two clades, form IA and form IC (Fig. 1). Of the bacterial *cbbL* sequences, 88% were grouped in clade IC. This clade encompassed seven subclusters (I to VII), of which subclusters II, III and VII contained 70% of the form IC *cbbL* clones and accounted for 28%, 19% and 23% of the total form IC *cbbL* genes, respectively. Except for subcluster II, their distribution between soils was uneven. For example, in subcluster III, about 70% of the clones were from upland soil, whereas in subcluster VII the clones were mainly from paddy soil. The remaining four subclusters contained only 30% of the form IC *cbbL* genes, and their distribution between soils did not appear to

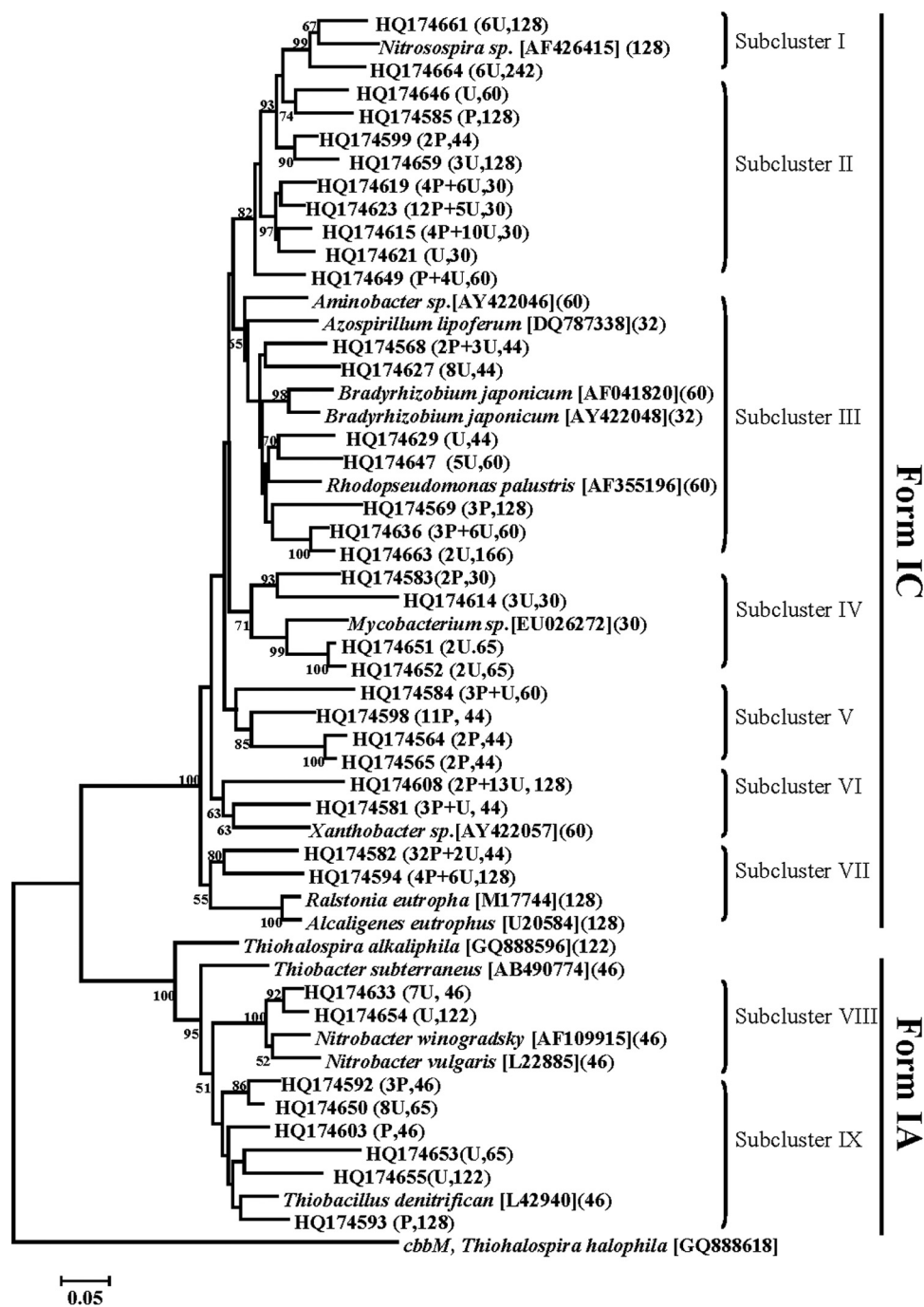


FIG 1 Phylogenetic tree of bacterial *cbbL* sequences from soils with the *cbbM* gene from *Thiohalospira halophila* (accession no. GQ888618) used as an outgroup. Bootstrap values (>50%) are indicated at the branch points. The paddy soil (P) and upland soil (U) are shown in brackets, and the numbers before each soil abbreviation represent the respective number of clones. The numbers after soil abbreviation indicate the respective sizes of the T-RFs after *in silico* analysis with HhaI. The scale bar represents a 5% estimated sequence divergence.

follow any specific pattern. Clones in subclusters I, IV, and VI were mostly recovered from the upland soil, while subcluster V clones were predominantly from paddy soil. In contrast to the form IC *cbbL* sequences, the form IA *cbbL* sequences were less diverse and formed only two subclusters, VIII and IX, which were mostly from the upland soil. Additionally, six of the nine subclusters grouped with the cultivated *cbbL*-containing bacteria. In the

form IC clade, the major subcluster III grouped with known sequences of *Azospirillum lipoferum* (DQ787338), *Aminobacter* sp. (AY422046), *Bradyrhizobium japonicum* (AY422048 and AF041820), and *Rhodopseudomonas palustris* (AF355196), and subcluster VII grouped with *Ralstonia eutropha* (M17744) and *Alcaligenes eutrophus* (EU20584). In the form IA clade, subcluster VIII included known *cbbL* sequences from *Nitrobacter winogradsky* (AF109915) and *Ni-*

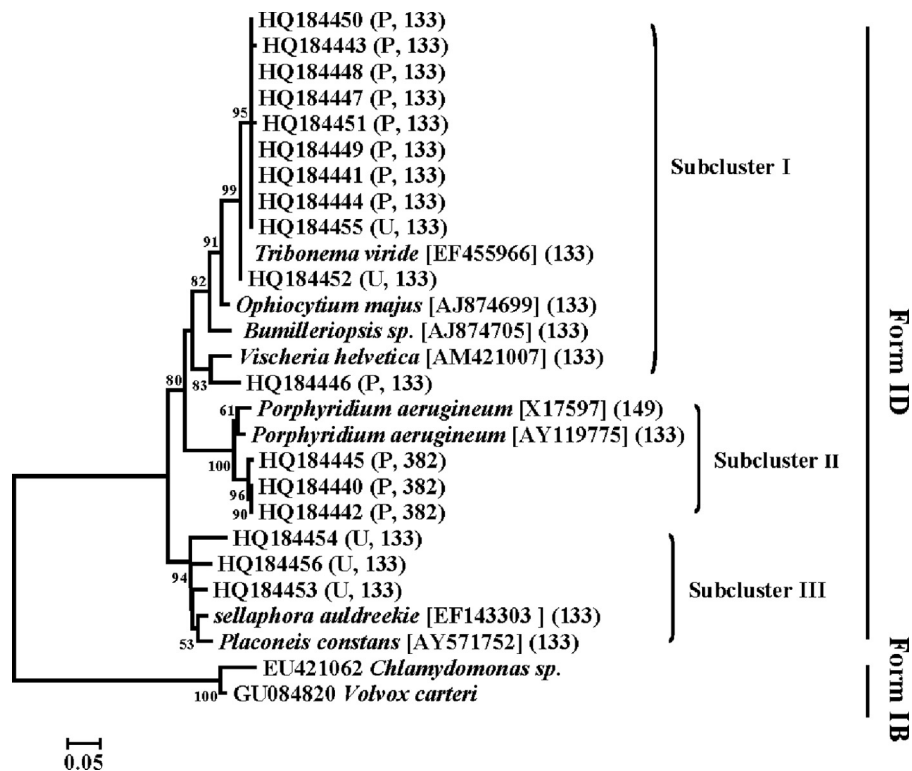


FIG 2 Phylogenetic tree of algal *cbbL* sequences from soils with the *Chlamydomonas* sp. (EU421062) and *Volvox carteri* (GU084820) used as outgroups. Bootstrap values (>50%) are indicated at the branch points. The paddy soil (P) and upland soil (U) are shown in brackets, and the numbers before each soil abbreviation represent the respective number of clones. The numbers after soil abbreviation indicate the respective sizes of the T-RFs after *in silico* analysis with RsaI. The scale bar represents a 5% estimated sequence divergence.

trobacter vulgaris (L22885) while subcluster IX included *Thiobacillus denitrificans* (L42940) gene sequences.

Amplified chromophytic algal *cbbL* fragments and related reference sequences formed three subclusters that demonstrated a relatively large diversity of chromophytic algae and included *Xanthophyta*, *Bacillariophyta*, and *Rhodophyta* (Fig. 2). Subclusters I and II accounted for 82% of the total and were dominated by clones from paddy soils. Subclusters I and II included known *cbbL* sequences from *Tribonema viride* (EF455966) and *Porphyridium aerugineum* (X17597 and AY119775), respectively. The remaining clade, subcluster III, contained only 18% of the total clones and included sequences primarily from upland soil. Comparison with known sequences showed similarities with *Sellaphora auldreekie* (EF143303) and *Placoneis constans* (AY571752). Overall, the phylogenetic analysis indicated that there were distinct differences in *cbbL* gene diversity between soils.

T-RFLP analyses of the *cbbL*-containing bacterial and algal community compositions. A total of 14 T-RFs were identified and used to compare bacterial *cbbL* communities (Fig. 3a). Soil type had an impact on both the presence and the relative abundance of different bacterial *cbbL* T-RFs. For example, the relative abundance of the T-RF of 30 bp in length (T-RF 30bp) was highest in soil U3 and lowest in soil P1 whereas T-RF 60bp was highest in U3 and lowest in P3 (Fig. 3a). T-RFs 46bp and 128bp were major components in soils U1 and U2, with relative abundances significantly higher than those in other soils except for P1 ($P < 0.05$). Some general trends in *cbbL* distribution were also evident between paddy and upland soils. The values for T-RF 30bp (22.1 to

35.5%) and T-RF 60bp (17.1 to 50.3%) in upland soils were generally higher than those of paddy soils (10.6 to 26.4% and 11.0 to 12.7%, respectively) and for T-RF 60bp the differences between soils were significant ($P < 0.05$). In contrast, T-RF 44bp was a major component of the *cbbL* gene diversity in all paddy soils but not in upland soils. Notwithstanding the limitations of extrapolating from TRFs to organism identity, the *in silico* analysis indicated that the dominant T-RFs 30bp, 60bp, 44bp, and 128bp were most closely related to facultatively autotrophic bacteria such as *Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, and *Ralstonia eutropha*. This is consistent with the results of the cloning analyses, where 88% of the cloned *cbbL* sequences clustered with RubisCO form 1C (facultative autotrophs) and with known sequences of *Bradyrhizobium*, *Rhodopseudomonas*, and *Ralstonia*. Such sequences were detected in all soils and comprised a major part of the *cbbL*-containing bacterial community. Obligate autotrophic bacterial *cbbL* gene sequences (form 1A), as represented by T-RF 46bp, from organisms such as *Thiobacillus denitrificans*, *Nitrobacter winogradskyi*, and *Nitrobacter vulgaris*, were a relatively small fraction of the autotrophic bacteria in these soils. Sequences representing these genera were also recovered in the clone library (Fig. 1), and the differences in relative abundance may indicate that bacterial CO₂ fixation in soils is more prevalent in facultative than in obligate autotrophs.

Comparison of 24 chromophytic algal *cbbL* T-RFLP profiles (Fig. 3b) showed that nine T-RFs (34bp, 42bp, 48bp, 52bp, 62bp, 64bp, 77bp, 133bp, and 285bp) presented as major components of the *cbbL* gene diversity in these soils. The relative abundance of

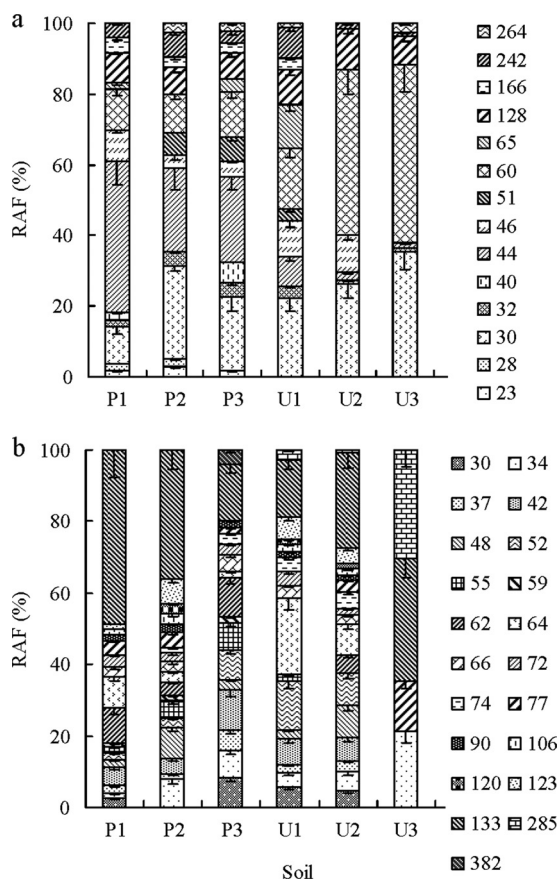


FIG 3 Average relative abundances of bacterial *cbbL* T-RFs (a) and algal T-RFs (b) from soils with light incubation. Different shadings on the graph have been used to indicate differences in fragment size. Bars indicate the standard errors of the means ($n = 4$).

these T-RFs varied markedly with soil type. For example, the T-RFs 42bp and 62bp were major components in paddy soils while the T-RFs 34bp, 48bp, 77bp, and 285bp dominated in upland soils. The T-RF 133bp was detected in all soils, and in general the relative abundance of T-RF 133bp in paddy soils (mean = 33.5%; coefficient of variation [CV] = 49%) was higher than in upland soils (mean = 25.6%; CV = 36%). *In silico* comparison of fragment length showed that only two of the T-RFs from these soils showed fragment lengths that were similar to those of *cbbL* sequences from known chromophytic algae. The 133bp fragment was similar to that from *Xanthophyta* and *Bacillariophyta*, while the 382bp fragment could be putatively identified as originating from *Rhodophyta*. These taxa are also represented in the clone library (Fig. 2).

The comparative analysis (CCA) showed that the compositions of *cbbL*-containing bacterial and algal communities in upland soils were clearly differentiated from each other while the compositions of the paddy soil samples were relatively close. The variation among different *cbbL*-containing bacterial and algal community compositions was most highly correlated with SOC (as determined at $P = 0.002$ using a Monte Carlo permutation test within the CCA analysis) (Fig. 4).

Abundances of bacterial and non-green-algal *cbbL* genes in soils. From the soils incubated in light, the bacterial *cbbL* abun-

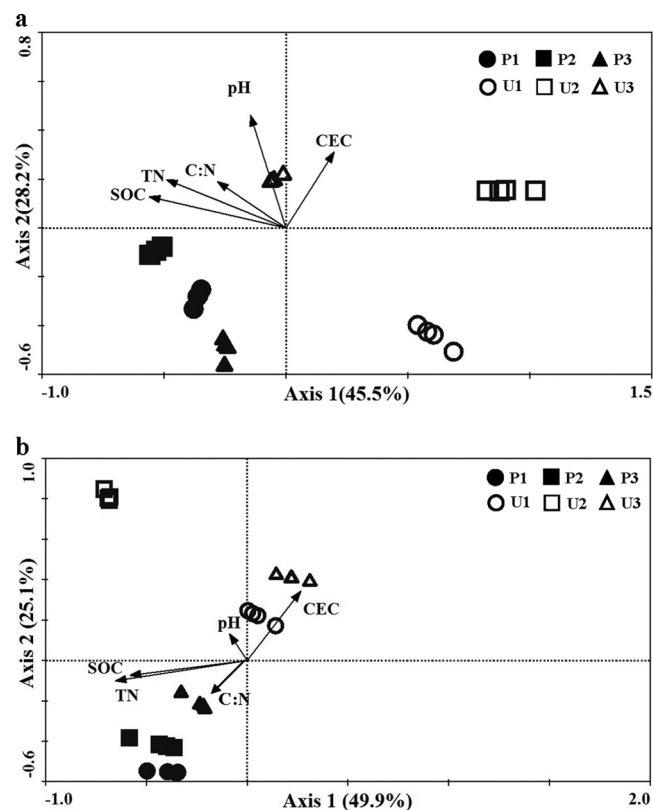


FIG 4 CCA analyses of the T-RFLP profiles for the bacterial (a) and chromophytic algal (b) *cbbL* gene diversity from the soils with light incubation. Arrows represent the environmental variables.

dance ranged from 4.2×10^6 to 1.25×10^8 copies g^{-1} soil and the chromophytic algal *cbbL* abundance ranged from 8.2×10^3 to 1.8×10^6 copies g^{-1} soil (Table 3). In general, the copy number of the bacterial *cbbL* was higher (14 to 1,964 times) than that of the chromophytic algal *cbbL*. Soil type was shown to have a significant effect on the abundance of bacterial and chromophytic algal *cbbL*, with paddy soils having a greater number (4 to 30 times) of bacterial *cbbL* copies than upland soils. The highest bacterial *cbbL* abundance was observed in P2 and P3, and the lowest was in U3. Although the copy numbers for the chromophytic algal *cbbL* gene

TABLE 3 Abundance of bacterial and chromophytic algal *cbbL* genes in the different soils

Soil ^a	Abundance ^b			
	Bacteria (10^8 copies g^{-1} soil)		Algae (10^6 copies g^{-1} soil)	
	Light	Dark	Light	Dark
P1	0.99 ± 0.02 B	0.59 ± 0.02	0.62 ± 0.06 B	0.37 ± 0.04
P2	1.17 ± 0.03 A	0.58 ± 0.03	1.39 ± 0.12 A	0.22 ± 0.01
P3	1.25 ± 0.02 A	0.62 ± 0.02	1.40 ± 0.27 A	0.56 ± 0.02
U1	0.22 ± 0.03 C	0.11 ± 0.02	1.08 ± 0.17 A	0.69 ± 0.04
U2	0.14 ± 0.01 D	0.07 ± 0.01	0.01 ± 0.00 C	ND
U3	0.04 ± 0.01 E	0.02 ± 0.00	0.01 ± 0.00 C	ND

^a See Table 1 for soils.

^b Means followed by the same letter are not significantly different ($P > 0.05$) between different soil types. In all cases, the mean with light incubation was significantly ($P < 0.05$) larger than that with dark incubation for the same soil. ND, not detectable.

TABLE 4 RubisCO activity in the soils incubated for 80 days

Soil ^a	RubisCO (nmol CO ₂ g ⁻¹ soil min ⁻¹) ^b	
	Light	Dark
P1	3.65 ± 0.40 C	0.58 ± 0.09
P2	6.73 ± 0.48 A	0.89 ± 0.17
P3	5.55 ± 0.76 B	1.66 ± 0.42
U1	3.29 ± 0.64 C	1.89 ± 0.27
U2	3.09 ± 0.29 C	ND
U3	0.77 ± 0.28 D	ND

^a See Table 1 for soils.

^b Means followed by the same letter are not significantly different ($P > 0.05$) between different soil types. The mean with light incubation was significantly ($P < 0.05$) larger than that with dark incubation for the same soil. ND, not detectable.

were generally lower, they also showed similar differences by soil type, with the highest chromophytic algal signals found in P3 and the lowest in U3. After 80 days of incubation, significantly lower *cbbL* abundances of bacteria and chromophytic algae were detected in soils incubated in the dark compared to those incubated in the light, suggesting that *cbbL* fixing potential declined when soils were incubated in the dark.

RubisCO enzyme activity in different soils. After 80 days of incubation, RubisCO activity was barely detectable in the soils incubated in the dark while significant RubisCO activity was measured in soils incubated in light. This activity ranged from 0.77 to 6.73 nmol CO₂ g⁻¹ soil min⁻¹ (Table 4). There were significant differences in RubisCO activity between soils. The highest RubisCO activity was measured in the paddy soil P2, with the lowest seen in the upland soil, U3. In general, measured RubisCO activities in paddy soils were higher than those in upland soils. Correlation analysis showed that the activity of RubisCO was closely related to the abundance of bacterial *cbbL* ($r = 0.853$; $P < 0.05$). However, no significant correlation was observed between RubisCO activity and chromophytic algal *cbbL* abundance ($P > 0.05$).

DISCUSSION

The work reported here shows that the diversity of bacterial and chromophytic algal *cbbL* genes encoding RubisCO in soils offers significant potential for the microbial assimilation of atmospheric CO₂. Carney et al. (4) considered that C emission and absorption in terrestrial ecosystems in response to global climate change do not balance. Thus, the quantity of CO₂ lost from soil is always greater than that resulting from CO₂ uptake. If correct, this suggests that there is a “missing C sink” of about 2 to 3 Pg C year⁻¹ at the global scale (14). Although the work reported here needs to be extended to include many more soils and soil types, the calculated rate of assimilation of organic C is approximately 0.013 to 0.103 g C m⁻² day⁻¹ (12 h light exposure). This would equate to a global rate for microbial synthesis of organic C of 4.9 to 37.5 g C m⁻², or 0.68 to 4.9 Pg per annum, assuming a total terrestrial area of 1.4×10^8 km². This means that microbial autotrophy could account for up to 4% of the total CO₂ fixed by terrestrial ecosystems each year. Since there was significant ¹⁴C₂ assimilation only when soils were incubated in the light, with almost no uptake when incubated in the dark, it seems reasonable to assume that the microbial CO₂ assimilation processes were predominantly phototrophic and, as such, were driven primarily by autotrophs (including photo- and chemoautotrophic microbes) rather than heterotrophs.

The measurement of RubisCO enzyme activity in these soils was shown to be closely related to the synthesis rate of SOC ($r = 0.946$; $P < 0.01$) and was positively correlated ($r = 0.853$; $P < 0.05$) with the abundance of the bacterial *cbbL* gene. There is no significant correlation with the abundance of the chromophytic algal genes supporting the hypothesis that facultatively autotrophic bacteria were the major microbial groups involved in CO₂ assimilation. The diversity and abundance of these bacteria differed between soils and were correlated with soil use and SOC content, suggesting that soil management and cropping regime might be manipulated to enhance soil C sequestration by enhancing the growth of facultatively anaerobic bacteria. This hypothesis is supported by previous investigations that also showed a link between bacterial *cbbL* gene diversity and local edaphic factors such as plant cover, land use, and fertilizer management (23, 33, 40). However, the dominant factors regulating the diversity of CO₂-fixing bacteria in soils and the potential for such systems to sequester carbon need further evaluation.

The work reported here has shown that the diversity and abundance of microbial autotrophs varies with soil type and land use. Of the physicochemical properties measured, the multivariate statistical analysis showed that the major influence on *cbbL* gene diversity and, by inference, on soil bacterial autotrophs is SOC. Soil organic matter is the most active fraction in soil and is a key determinant of soil fertility and productivity (9, 25, 42). SOC also stabilizes soil structure (7) and is an important source of carbon for microbial growth and of nutrients such as phosphorus, sulfur, calcium, magnesium, and trace elements (30). The finding that total gene copy number (qPCR) was lower in soils with low levels of SOC could be due to the limited availability of the C needed to support growth, since facultatively autotrophic bacteria, and not obligate autotrophs, were the more dominant (32, 33). The size of the *cbbL*-containing chromophytic algal community was related in a similar way to SOC levels. The algal *cbbL* sequences recovered in this study belonged to the genera *Xanthophyta* and *Bacillariophyta*. Although the algae were typically autotrophic organisms, some were mixotrophs and as such derive energy from both photosynthesis and the uptake of organic carbon either by osmotrophy, mixotrophy, or phagotrophy (18). In soils, the decomposition of soil organic matter can provide carbon dioxide for algal photosynthesis, with the transformation of soil organic matter providing rich organic substrates and key nutrients for mixotrophic algal growth. These findings are in general agreement with recent studies on nutrient dynamics and their impact on microbial community structure of bacteria and phytoplankton in different ecosystems (11, 32).

Since management can be used to moderate SOC levels in soils, the interaction between SOC levels and the CO₂ assimilation activities of facultatively autotrophic bacteria warrants further investigation. For example, *Ralstonia eutropha* and *Alcaligenes eutrophus* were identified both in the clone libraries (Fig. 1, subcluster VII) and from the T-RFLP analyses as significant components of the microbial community in paddy soils. *Ralstonia eutropha* and *Alcaligenes eutrophus* are known to produce and sequester polyhydroxyalkanoates that can accumulate intracellularly to approximately 90% of the cell's dry weight (44). These polyhydroxyalkanoates are not readily turned over in flooded soils and are largely protected from mineralization in paddy soils by complexation with active iron oxides. This capacity for organic matter complexation and protection is considerably reduced in

upland soils, where the amounts of active iron are generally lower. Taken together, the increase in RubisCO activity, the increase in facultative autotrophic bacteria, and the production of highly stable forms of soil carbon (polyhydroxyalkanoate) make paddy soils and other inundated systems potential land use options for maximizing the retention and sequestration of atmospheric CO₂. However, additional work is needed to confirm the link between the changes in the relative abundance of *cbbL* genes and the capacity for paddy soils to fix CO₂. These studies include the need for more detailed isotope tracer studies (13) and an investigation of alternate CO₂ assimilation pathways such as the 3-hydroxypropionate cycle, the reductive citric acid cycle, and the reductive acetyl-CoA pathway (8, 12, 20).

A combination of RubisCO activity measurements, *cbbL* gene diversity analysis, and real-time qPCR has highlighted the potential for cropping systems (e.g., paddy and upland) soils to sequester CO₂. These studies also suggest that the role of facultatively autotrophic bacteria and chromophytic algae in the biogeochemical cycling of soil C has probably been underestimated. T-RFLP and sequence analysis of clone libraries have shown that the major T-RF components among the bacterial autotrophs were indicative of taxa known to accumulate significant amounts of stable C in the form of polyhydroxyalkanoates and indicate that land management in arable and paddy systems that optimizes facultatively autotrophic activity could be a viable option for enhancing the sequestration and stabilization of carbon in soils.

ACKNOWLEDGMENTS

This study was jointly supported by the Knowledge Innovation Program of the Chinese Academy of Sciences (grant no. KZCX3-SW-437, ISACX-LYQY-QN-1103), the Strategic Priority Research Program–Climate Change: Carbon Budget and Related Issues of the Chinese Academy of Sciences (grant no. XDA05050505), the National Natural Science Foundation of China (40901124, 41090283), and the International S&T cooperation program of China (grant no. 2011DFA30770).

REFERENCES

- Altschul SF, et al. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389–3402.
- Bell JM, Falconer C, Colby J, Williams E. 1987. CO metabolism by a thermophilic actinomycete, *Streptomyces* strain G26. *J. Gen. Microbiol.* 133:3445–3456.
- Butterly CR, McNeill AM, Baldock JA, Marschner P. 2011. Rapid changes in carbon and phosphorus after rewetting of dry soil. *Biol. Fertil. Soils* 47:41–50.
- Carney KM, Hungate BA, Drake BG, Megonigal JP. 2007. Altered soil microbial community at elevated CO₂ leads to loss of soil carbon. *Proc. Natl. Acad. Sci. U. S. A.* 104:4990–4995.
- Chen Z, et al. 2010. Impact of long-term fertilization on the composition of denitrifier communities based on nitrite reductase analysis in a paddy soil. *Microb. Ecol.* 60:850–861.
- Falkowski P, et al. 2000. The global carbon cycle: a test of our knowledge of earth as a system. *Science* 290:291–296.
- Fonte SJ, Barrios E, Six J. 2010. Earthworms, soil fertility and aggregate-associated soil organic matter dynamics in the Quesungual agroforestry system. *Geoderma* 155:320–328.
- Fuchs G. 1989. Alternative pathways of autotrophic CO₂ fixation, p 365–382. *In* Schlegel HG, Bowien B (ed), *Autotrophic bacteria*. Springer-Verlag KG, Berlin, Germany.
- Ge T, et al. 2011. Chemical properties, microbial biomass, and activity differ between soils of organic and conventional horticultural systems under greenhouse and open field management: a case study. *J. Soils Sediment.* 11:25–36.
- Hanson TE, Tabita FR. 2001. A ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)-like protein from *Chlorobium tepidum* that is involved with sulfur metabolism and the response to oxidative stress. *Proc. Natl. Acad. Sci. U. S. A.* 98:4397–4402.
- Heil CA, Revilla M, Glibert PM, Murasko S. 2007. Nutrient quality drives differential phytoplankton community composition on the southwest Florida shelf. *Limnol. Oceanogr.* 52:1067–1078.
- Holo H. 1989. *Chloroflexus aurantiacus* secretes 3-hydroxypropionate, a possible intermediate in the assimilation of CO₂ and acetate. *Arch. Microbiol.* 151:252–256.
- Jenkins SN, et al. 2010. Taxon-specific responses of soil bacteria to the addition of low level C inputs. *Soil Biol. Biochem.* 42:1624–1631.
- Karim A, Veizer J, Barth J. 2008. Net ecosystem production in the Great Lakes basin and its implications for the North American missing carbon sink: a hydrologic and stable isotope approach. *Global Planet. Change* 61:15–27.
- Kellogg EA, Juliano ND. 1997. The structure and function of RuBisCO and their implications for systematic studies. *Am. J. Bot.* 84:413–428.
- Kusian B, Bowien B. 1997. Organization and regulation of *cbb* CO₂ assimilation genes in autotrophic bacteria. *FEMS Microbiol. Rev.* 21:135–155.
- Lal R. 2008. Carbon sequestration. *Philos. Trans. R. Soc. B.* 363:815–830.
- Litchman E, Klausmeier CA. 2008. Trait-based community ecology of phytoplankton. *Annu. Rev. Ecol. Evol. Syst.* 39:615–639.
- Lukow T, Dunfield PF, Liesack W. 2000. Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure with in an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiol. Ecol.* 32:241–247.
- Menendez C, et al. 1999. Presence of acetyl coenzyme A (CoA) carboxylase and propionyl-CoA carboxylase in autotrophic Crenarchaeota and indication for operation of a 3-hydroxypropionate cycle in autotrophic carbon fixation. *J. Bacteriol.* 181:1088–1098.
- Mouritzen P, et al. 2005. ProbeLibrary: a new method for faster design and execution of quantitative real-time PCR. *Nat. Methods* 2:313–316.
- Müller T, Höper H. 2004. Soil organic matter turnover as a function of the soil clay content: consequences for model applications. *Soil Biol. Biochem.* 36:877–888.
- Nanba K, King GM, Dunfield K. 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.
- O'Donnell AG, Falconer C, Goodfellow M, Ward AC, Williams E. 1993. Biosystematics and diversity amongst novel carboxydutrophic actinomycetes. *Antonie Van Leeuwenhoek* 64:325–340.
- O'Donnell AG, Young IM, Rushton SP, Shirley MD, Crawford JW. 2007. Visualization, modelling and prediction in soil microbiology. *Nat. Rev. Microbiol.* 5:689–699.
- Pachauri RK, Reisinger A. 2007. Climate change 2007: synthesis report. Intergovernmental Panel on Climate Change, Cambridge, United Kingdom.
- Paul J, Alfreider A, Wawrik B. 2000. Micro- and macrodiversity in *rbcl* sequences in ambient phytoplankton populations from the southeastern Gulf of Mexico. *Mar. Ecol. Prog. Ser.* 198:9–18.
- Rhoades JD. 1982. Cation exchangeable capacity, p 149–165. *In* Pace AL, Miller RH, Keeney DR (ed), *Methods of soil analysis, part 2: chemical and microbiological properties*, 2nd ed. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI.
- Sambrook J, Russell DW. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Saul-Tcherkas V, Steinberger Y. 2009. Substrate utilization patterns of desert soil microbial communities in response to xeric and mesic conditions. *Soil Biol. Biochem.* 41:1882–1893.
- Savage DF, Afonso B, Chen AH, Silver PA. 2010. Spatially ordered dynamics of the bacterial carbon fixation machinery. *Science* 327:1258–1261.
- Selesi D, Schmid M, Hartmann A. 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.
- Selesi D, Pattis I, Schmid M, Kandeler E, Hartmann A. 2007. Quantification of bacterial RubisCO genes in soils by *cbbL* targeted real-time PCR. *J. Microbiol. Methods* 69:497–503.
- Siegenthaler U, Sarmiento JL. 1993. Atmospheric carbon dioxide and the ocean. *Nature* 365:119–125.

35. Tabita FR. 1999. Microbial ribulose-1,5-bisphosphate carboxylase/oxygenase: a different perspective. *Photosynth. Rev.* **60**:1–28.
36. Takai K, et al. 2005. Enzymatic and genetic characterization of carbon and energy metabolisms by deep-sea hydrothermal chemolithoautotrophic isolates of *Epsilonproteobacteria*. *Appl. Environ. Microbiol.* **71**:7310–7320.
37. Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**:1596–1599.
38. Tcherkez GGB, Farquhar GD, Andrews TJ. 2006. Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. *Proc. Natl. Acad. Sci. U. S. A.* **103**:7246–7251.
39. ter Braak CJF. 1996. Unimodal models to relate species to environment. Agricultural Mathematics Group, Wageningen, The Netherlands.
40. Tolli J, King GM. 2005. Diversity and structure of bacterial chemolithotrophic communities in pine forest and agroecosystem soils. *Appl. Environ. Microbiol.* **71**:8411–8418.
41. Vance ED, Brookes PC, Jenkinson DS. 1987. Microbial biomass measurements in forest soils: determination of *kc* values and tests of hypotheses to explain the failure of the chloroform fumigation-incubation method in acid soils. *Soil Biol. Biochem.* **19**:689–696.
42. Wu J. 2011. Carbon accumulation in paddy ecosystems in sub-tropical China: evidence from landscape studies. *Eur. J. Soil Sci.* **62**:29–34.
43. Wu J, O'Donnell AG. 1997. Procedure for the simultaneous analysis of total and radioactive carbon in soil and plant materials. *Soil Biol. Biochem.* **29**:199–202.
44. Yabuuchi E, Kosako Y, Yano I, Hotta H, Nishiuchi Y. 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiol. Immunol.* **39**:897–904.