APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2005, p. 175–184 Vol. 71, No. 1
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.1.175–184.2005

Diversity of Green-Like and Red-Like Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Large-Subunit Genes (*cbbL*) in Differently Managed Agricultural Soils

Draženka Selesi, Michael Schmid, and Anton Hartmann*

Institute of Soil Ecology, GSF-National Research Centre for Environment and Health, Neuherberg/Munich, Germany

Received 8 April 2004/Accepted 24 August 2004

A PCR-based approach was developed to detect ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) form I large-subunit genes (*cbbL*) as a functional marker of autotrophic bacteria that fix carbon dioxide via the Calvin-Benson-Bassham cycle. We constructed two different primer sets, targeting the green-like and red-like phylogenetic groups of *cbbL* genes. The diversity of these *cbbL* genes was analyzed by the use of three differently managed agricultural soils from a long-term field experiment. *cbbL* gene fragments were amplified from extracted soil DNAs, and PCR products were cloned and screened by restriction fragment length polymorphism analysis. Selected unique *cbbL* clones were sequenced and analyzed phylogenetically. The green-like *cbbL* sequences revealed a very low level of diversity, being closely related to the *cbbL* genes of *Nitrobacter winogradskyi* and *Nitrobacter vulgaris*. In contrast, the red-like *cbbL* gene libraries revealed a high level of diversity in the two fertilized soils and less diversity in unfertilized soil. The majority of environmental red-like *cbbL* genes were only distantly related to already known *cbbL* sequences and even formed separate clusters. In order to extend the database of available red-like *cbbL* sequences, we amplified *cbbL* sequences from bacterial type culture strains and from bacterial isolates obtained from the investigated soils. Bacterial isolates harboring the *cbbL* gene were analyzed phylogenetically on the basis of their 16S rRNA gene sequences. These analyses revealed that bacterial genera such as *Bacillus*, *Streptomyces*, and *Arthrobacter* harbor red-like *cbbL* genes which fall into the *cbbL* gene clusters retrieved from the investigated soils.

The Calvin-Benson-Bassham cycle is the major and most abundant pathway for CO_2 fixation (30). In addition to its essential capacity as the mechanism of primary production in nearly all ecosystems, the Calvin cycle plays a major role in effecting the concentration of atmospheric CO_2. The Calvin cycle exists in diverse organisms, from bacteria to algae to green plants. The most abundant protein on earth, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), catalyzes the first, rate-limiting step in the Calvin cycle (5). RubisCO is a bifunctional enzyme that controls the reduction of CO_2 and the oxygenolysis of ribulose-1,5-bisphophate. Since RubisCO is responsible for the overwhelming amount of carbon fixation by plants, nearly all primary production is linked to the function of this enzyme. RubisCO is a well-studied enzyme because of its extensive agricultural and environmental significance (2, 14, 22).

RubisCO exists in multiple natural forms which differ in structure, catalytic property, and O_2 sensitivity (33). Form I RubisCO is a hexadecamer composed of eight large and eight small subunits (L_8S_8) and occurs in photo- and chemoautotrophic organisms. The form II protein consists only of large subunits (L_n), with 25 to 30% amino acid sequence identity to form I (33), and is found in photo- and chemoautotrophs. It is assumed that the common ancestor of RubisCO was similar to the form II enzyme because it operates successfully under low-O_2 and high-CO_2 concentrations, which resemble the conditions that existed in the early earth's atmosphere (12, 33). The form I RubisCO protein evolved as the atmospheric CO_2 concentration decreased and the O_2 concentration increased (18, 27). Recently, it was discovered that some members of *Archaea* possess a form III RubisCO (3, 16). Forms I, II, and III of RubisCO contain catalytic active amino acid residues that are necessary for carboxylation as well as oxygenation (8). Form IV RubisCO, which lacks several of the required amino acid residues for the catalytic activity of RubisCO (8), has been discovered in *Bacillus subtilis* (17), *Chlorobium tepidum* (8), and *Archaeoglobus fulgidus* (16). Form IV is designated a RubisCO-like protein, as its sequence is most closely related to RubisCO but it is not involved in the Calvin cycle.

The large subunit of form I RubisCO is encoded by the *cbbL* gene (18). The *cbbL* gene is 1,400 bp long and thus is large enough for use in meaningful phylogenetic analyses, for which there are sufficient sequences of *cbbL* genes deposited in public databases. Phylogenetic studies based on these *cbbL* sequences revealed that form I RubisCO proteins can be subdivided into two major groups, the green-like and red-like groups (36). The green-like group contains *cbbL* sequences from plants, algae, and α-, β-, and γ-*Proteobacteria* as well as from *Cyanobacteria*. The red-like type occurs in nongreen algae and α- and β-*Proteobacteria.* Form I RubisCO can be considered an enzyme that is predominantly found in photosynthetic and aerobic chemolithoautotrophic organisms. Clearly, the *cbbL* phylogeny disagrees with the phylogeny based on rRNA gene sequences (4). Thus, for instance, *Rhodobacter capsulatus* contains a green-like *cbbL* gene, whereas *Rhodobacter*

* Corresponding author. Mailing address: GSF-National Research Center for Environment and Health, Institute of Soil Ecology, Department of Rhizosphere Biology, Ingolstädter Landstrasse 1, D-85764 Neuherberg/Munich, Germany. Phone: 49 89 3187-4109. Fax: 49 89 3187-3376. E-mail: anton.hartmann@gsf.de.

TABLE 1. PCR amplification of genomic DNAs from reference organisms by the use of selected *cbbL* primer pairs

cbbL type	Species	Strain[a]	PCR result[b]	Presence of database sequence[c]
Red-like	*Azospirillum brasilense* Sp 7	ATCC 29145	−	−
	Azospirillum lipoferum	GSF 19	+	−
	Azospirillum doebereinerae	GSF 21	−	−
	Herbaspirillum seropedicae	DSM 6445	−	−
	Ochrobactrum anthropi	DSM 6882	+	−
	Ochrobactrum tritici	DSM 13340	−	−
	Ralstonia eutropha	DSM 531	+	+
	Ralstonia picketti	DSM 6297	−	−
	Rhizobium leguminosarum bv. trifolii	ATCC 53912	+	−
	Sinorhizobium fredii	ATCC 35423	+	−
	Sinorhizobium meliloti	DSM 30135	+	+
	Sinorhizobium terangae	DSM 11282	+	−
	Sinorhizobium xinjiangense	DSM 5852	−	−
	Xanthobacter agilis	DSM 3770	+	−
	Xanthobacter autotrophicus	DSM 432	+	−
	Xanthomonas campestris	DSM 1350	−	−
Green-like	*Nitrobacter vulgaris*	DSM 10236	+	+
	Nitrobacter winogradskyi	DSM 10237	+	+

[a] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; GSF, National Research Center for Environment and Health.
[b] +, PCR product of expected size; −, no amplification.
[c] Data are from the literature.

sphaeroides harbors a red-like enzyme. Additionally, both organisms have a closely related form II RubisCO (4). Studies with *Rhodobacter azotoformans* demonstrated for the first time that a single bacterial cell can contain green-like as well as red-like *cbbL* genes (34).

Microorganisms play a key role in our understanding of regional and global carbon ecology, as they are involved in almost all processes of the carbon cycle due to their abundance and high metabolic diversity. Soils are significant compartments of the terrestrial carbon cycle and act as a source or sink for different carbon compounds, such as CO_2 or methane. However, the diversity of CO_2 fixation in soil bacteria has not yet been studied in detail. We focused on the apparently most important functional gene (*cbbL*) involved in CO_2 fixation and developed distinct *cbbL*-specific primer sets to detect bacterial green-like and red-like types of the large-subunit gene of form I RubisCO. The aim of this study was to determine the diversity and composition of these *cbbL* types in soil bacteria isolated from differently managed agricultural soils from a long term, so-called eternal rye experiment in Halle/Saale, Germany. For a period of more than 125 years, these soils have been planted with rye and have not received fertilizer, mineral fertilizer (N, P, or K), or farmyard manure.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used for this study are listed in Table 1. They were cultured as recommended by the Deutsche Sammlung von Mikroorganismen und Zellkulturen type culture collection (Braunschweig, Germany). These bacterial cultures were used to evaluate the specificities of the newly designed *cbbL* primers and to check for the presence of *cbbL* genes.

Soil samples. Topsoil samples (from a depth of 0 to 10 cm) were taken in autumn 2000 from an agricultural long-term field experiment in Halle (Saale), Germany (eternal rye cultivation). The soil is a typical haplic phaeozem, and the matrix consists of 8% clay, 23% silt, and 69% sand (23). The experiment started in 1878 and exploits the sustainable applicability of different fertilization treatments on the cultivation of rye. For this study, samples from the following three treatment groups were analyzed: (i) HKO, a plot that remained unfertilized; (ii) HSM, a plot that received farmyard manure (12 tons ha^{-1} $year^{-1}$); and (iii) HNPK, a plot that received mineral fertilizer (60 kg of N, 24 kg of P, and 75 kg of K ha^{-1} $year^{-1}$). After sampling, soil samples were passed through a 2-mm-pore-size sieve to remove plant material. DNA extraction was performed immediately, or samples were frozen at −20°C after sample collection.

Isolation of bacterial strains. In order to recover soil bacteria with hitherto unknown red-like *cbbL* genes, we isolated bacteria from the HNPK soil sample. One gram of soil was mixed with 9 ml of an extraction solution (0.1 g of NaCl $liter^{-1}$, 0.02 g of $CaCl_2 \cdot 2H_2O$ $liter^{-1}$, 0.2 g of $MgSO_4 \cdot 7H_2O$ $liter^{-1}$, 5 g of Tween 80 $liter^{-1}$) and homogenized for 5 min in an oscillating mixer (Retsch, Haan, Germany). The soil suspension was serially diluted to a factor of 10^{-6}. Aliquots (100 μl) were spread on *Rhizobium* medium (21) and incubated for 2 days at 22°C. Sixty-four colonies were picked randomly, streaked on *Rhizobium* medium, and incubated for 2 days at 30°C. For DNA extraction, colonies from bacterial isolates were cultured in 3 ml of liquid *Rhizobium* medium overnight at 30°C. The cells were centrifuged and used for DNA extraction as described below.

Primer design. All *cbbL* nucleotide sequences which were available from the National Center for Biotechnology Information sequence database were used to establish a *cbbL* database by use of the ARB software package (20; http://www.arb-home.de). The sequences were translated into amino acids, and the deduced amino acid sequences were aligned with the GDE sequence editor implemented in the ARB software package. Amino acid alignments were performed manually, and nucleotide sequences were aligned accordingly. Based on these data, we designed two primer sets specific for selected *cbbL* sequences of the red-like and green-like groups. The primers *cbbL*R1F and *cbbL*R1R, which were used for amplification of the red-like RubisCO form I *cbbL* gene, were designed from sequence alignment data given for the *cbbL* genes of *Ralstonia eutropha* H16, the *Ralstonia eutropha* megaplasmid pHG1, and *Sinorhizobium meliloti* WSM419. The primers *cbbL*G1F and *cbbL*G1R, which were used for amplification of the green-like *cbbL* genes, were designed from multiple sequence alignment data for the *cbbL* genes of *Nitrobacter vulgaris* T3, *Nitrobacter winogradskyi* IFO14297, *N. winogradskyi* ATCC 14123, *Hydrogenophaga pseudoflava* DSM1083, *Thiobacillus denitrificans* ATCC 25259, and *Nitrospira* sp. strain TCH716. The primers that were designed and used for this study are listed in Table 2.

Extraction of chromosomal DNAs from soil. Genomic DNAs from pure bacterial cultures and soil samples were extracted and purified by use of a FastDNA spin kit for soil (Qbiogene Inc., Carlsbad, Calif.) according to the manufacturer's protocol.

Amplification of *cbbL* genes. Amplification of the RubisCO genes from extracted DNAs via PCR was performed with the newly designed primer pairs described above. Amplification from 100 ng of extracted DNA was performed in 50-μl reaction mixtures containing 50 pmol each of a forward and reverse primer, a 200 μM concentration of each deoxynucleoside triphosphate (Fermentas GmbH, St. Leon-Rot, Germany), 1.5 mM $MgCl_2$, and 1 U of *Taq* polymerase (Fermentas GmbH) in the 1× reaction buffer provided with the enzyme. The cycle conditions for *cbbL*-specific PCRs were as follows: 4 min of initial denaturation at 95°C, followed by 32 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 57°C for the red-like and 62°C for the green-like *cbbL* primers, and 1 min of elongation at 72°C. The reaction was completed by a final extension for 10 min at 72°C. Aliquots of the PCR products were analyzed in 1.5% (wt/vol) agarose gels (PeqLab Biotechnology GmbH, Erlangen, Germany) by horizontal gel electrophoresis. DNAs were visualized by UV excitation after staining with ethidium bromide (0.5 mg $liter^{-1}$).

Cloning and screening of environmental clones. PCR products of the expected sizes (1,100 bp for green-like and 800 bp for red-like genes) from soil samples as

TABLE 2. Selected primers used for amplification of *cbbL* genes

Primer[a]	Positions[b] (nt)	Primer sequence (5′-3′)[c]
*cbbL*R1F	634–651	AAG GAY GAC GAG AAC ATC
*cbbL*R1R	1435–1454	TCG GTC GGS GTG TAG TTG AA
*cbbL*G1F	397–416	GGC AAC GTG TTC GGS TTC AA
*cbbL*G1R	1413–1433	TTG ATC TCT TTC CAC GTT TCC

[a] Primers were named so that *cbbL*R1 primers targeted genes with red-like *cbbL* sequences and *cbbL*G1 primers targeted genes with green-like *cbbL* sequences; forward and reverse primers are indicated with an "F" or "R" as the last letter.
[b] Positions correspond to the red-like *cbbL* gene of *Ralstonia eutropha* H16 (U20584) and the green-like *cbbL* gene of *N. vulgaris* (L22885).
[c] Y = C or T; S = G or C.

well as from bacterial cultures were eluted from agarose gels by use of a NucleoSpin extraction kit (Macherey & Nagel, Düren, Germany). Eluted PCR products were ligated into the vector pCR2.1-TOPO (Invitrogen, San Diego, Calif.) and transformed into competent *Escherichia coli* cells provided with a TA cloning kit (Invitrogen) according to the manufacturer's protocol. Plasmids from the *cbbL* libraries were isolated by use of a NucleoSpin plasmid kit (Macherey & Nagel). Clones containing putative *cbbL* genes were screened by EcoRI restriction endonuclease digestion. Each 10-μl digestion reaction mixture consisted of 2 μl of purified plasmid, 1 μl of buffer O^+ (Fermentas GmbH), and 2 U of EcoRI (Fermentas GmbH) and was incubated at 37°C for 2 h. Clones which harbored a correctly sized red-like *cbbL* insert were screened by restriction fragment length polymorphism (RFLP). Ten microliters of each PCR product was hydrolyzed with 2 U of the restriction endonuclease BbvI (MBI Fermentas). Restriction fragments were analyzed in 3.5% (wt/vol) agarose gels (PeqLab) and visualized as described previously.

Amplification and cloning of 16S rRNA genes. To obtain the corresponding 16S rRNA gene sequences of *cbbL*-positive bacterial isolates, we performed PCRs to amplify the 16S rRNA gene from each isolate by using the primer pair 616-Forward (5′-AGA-GTT-TGA-TYM-TGG-CTC-AG-3′) and 630-Reverse (5′-CAK-AAA-GGA-GGT-GAT-CC-3′) (13), resulting in a full-length PCR product of about 1,500 bp. The PCR products were eluted from agarose gels and cloned as described above. Plasmids were extracted, and the presence of inserts with the correct size was checked as described above.

Sequencing reactions. Plasmids containing *cbbL* inserts from soil DNA or from pure bacterial cultures as well as 16S rRNA gene inserts from pure cultures were used directly for sequencing. Both strands were sequenced by use of the vector-specific primers M13 reverse and T7 promoter. The plasmids were sequenced in an ABI Prism 377 automated sequencer (Applied Biosystems, Weiterstadt, Germany) by use of a Big Dye Terminator sequencing kit (Applied Biosystems).

Phylogenetic analysis. The newly obtained *cbbL* nucleotide sequences were added to the established *cbbL* database implemented in the ARB software package (20; http://www.arb-home.de). The sequences were translated into amino acids, and the deduced amino acid sequences were aligned with GDE 2.2 editor software. Nucleic acid sequences were aligned according to the amino acid alignments. Phylogenetic analyses based on amino acid and nucleotide sequences were performed by applying maximum likelihood, maximum parsimony, and neighbor-joining methods by use of the respective tools in the ARB software package. The 16S rRNA gene sequences obtained from the isolates were added to an existing database of about 20,000 small-subunit rRNA gene sequences by use of the fast alignment tool of the ARB software package. Alignments were checked visually. Phylogenetic analyses based on 16S rRNA gene sequences were performed by the methods described above.

Statistical analysis of red-like *cbbL* libraries. To evaluate richness and evenness, we calculated diversity indices for the red-like *cbbL* libraries by using different patterns from the RFLP analysis as representations of different operational taxonomic units (OTUs) in a sample. The diversity indices included (i) species richness (S), or the total number of OTUs; (ii) library coverage (C), or the portion of a clone library of infinite size that was sampled (6); (iii) the Shannon-Weaver diversity index, calculated by use of the equation $H = -\Sigma(p_i)(\log_2 p_i)$, where p is the proportion of an individual OTU relative to the total number of all RFLP patterns (29); (iv) the Simpson's index, calculated by use of the equation $D = 1 - \Sigma(p_i)^2$ (32); and (v) evenness, calculated from the Shannon-Weaver diversity function by use of the equation $E = H/H_{max}$, where $H_{max} = \log_2 S$. The diversity of the clones was analyzed by rarefaction analysis (31). Rarefaction curves were produced by use of the analytical approximation algorithm described by Hurlbert (11), and 95% confidence intervals were estimated as described by Heck et al. (9). Calculations were performed with the Analytic Rarefaction freeware program (http://www.uga.edu/~strata/software/Software.html).

Nucleotide sequence accession numbers. The sequences determined in this study are available at GenBank under accession no. AY572110 to AY572155 (red-like *cbbL* sequences from environmental clones), AY572169 to AY572192 (green-like *cbbL* sequences from environmental clones), AY572156 to AY572168 (*cbbL* sequences from isolates), AY572464 to AY572473 (*cbbL* sequences from reference strains), and AY572474 to AY572486 (16S rRNA gene sequences from isolates).

RESULTS

Primer design. The high degree of variability of *cbbL* sequences made it impossible for us to design a universal PCR primer set to target all *cbbL* genes that were available in public databases. Sequence similarities calculated from distance matrices of all pairwise comparisons of *cbbL* nucleotide sequences ranged from 22 to 100%. The sequence similarities within the two *cbbL* clusters, the red-like group (57.8 to 100%) and the green-like group (60.7 to 99.2%), were significantly higher. Thus, we were able to construct primer sets that were specific for selected *cbbL* sequences of the two distantly related red-like and green-like *cbbL* clusters (Table 2). The red-like primers were derived from *cbbL* sequences of *Ralstonia eutropha* H16, *Ralstonia eutropha* megaplasmid pHG1, and *S. meliloti* WSM419, and the green-like primers were derived from *cbbL* sequences of *N. vulgaris* T3, *N. winogradskyi* strain IFO14297, *N. winogradskyi* strain ATCC 14123, *H. pseudoflava* DSM1083, *T. denitrificans* ATCC 25259, and *Nitrospira* sp. strain TCH716. Comparisons of the chosen primer sequences to all sequences available in public sequence databases indicated significant sequence similarities to *cbbL* genes only.

Amplification of *cbbL* genes from pure cultures and environmental samples. To evaluate the efficiency of the newly designed *cbbL*-specific primers, we performed gene amplification with DNAs extracted from *Ralstonia eutropha* and *S. meliloti* as representatives of the red-like group. Amplification with the primer combination *cbbL*R1F and *cbbL*R1R yielded the expected 800-bp size and generated specific products which were visible as a single band on stained agarose gels. We also successfully amplified the 800-bp *cbbL* gene fragment from DNAs extracted from all three different soil samples.

The efficiency of the green-like gene-specific *cbbL* primers *cbbL*G1F and *cbbL*G1R was determined by the use of reference DNAs extracted from *N. vulgaris* and *N. winogradskyi*. A distinct gene fragment of 1,100 bp was obtained. In contrast to amplification with the red-like gene-specific primers, positive amplification of RubisCO genes was only possible with DNAs from the HKO and HNPK soil samples. No PCR products were observed with DNAs from the HSM soil sample, which had received fertilization with farmyard manure every year. In order to exclude the effect of inhibiting contaminants in the soil, we performed the green-like gene-specific PCR with DNAs from HSM soil spiked with DNA from *N. vulgaris*. Since the obtained PCR product showed the expected *cbbL* fragment size of about 1,100 bp, inhibition effects could be excluded. Various efforts to optimize the PCR conditions were not successful at yielding the expected fragment size of green-like *cbbL* sequences from HSM soil.

To extend the data set of available red-like *cbbL* genes, we amplified *cbbL* genes from different bacterial strains from culture collections (Table 1). *cbbL* sequences from *Xanthobacter agilis* DSM3770, *Xanthobacter autotrophicus* DSM432, *Ochrobactrum anthropi* DSM6882 (19), *Sinorhizobium fredii* ATCC35423, *Sinorhizobium terangae* DSM11282, *Rhizobium leguminosarum* bv. Trifolii ATCC 53912, and *Azospirillum lipoferum* GSF19 were determined. Moreover, *cbbL* gene fragments of bacterial isolates from the HNPK soil sample were amplified by use of the red-like *cbbL* primer set. A total of 13 of 64 investigated bacterial isolates revealed the correct PCR product size of 800 bp.

Green-like *cbbL* clone libraries. PCR products with the correct size that were amplified from the HKO and HNPK soil samples by the use of green-like gene-specific *cbbL* primers were used to establish clone libraries. A total of 59 clones

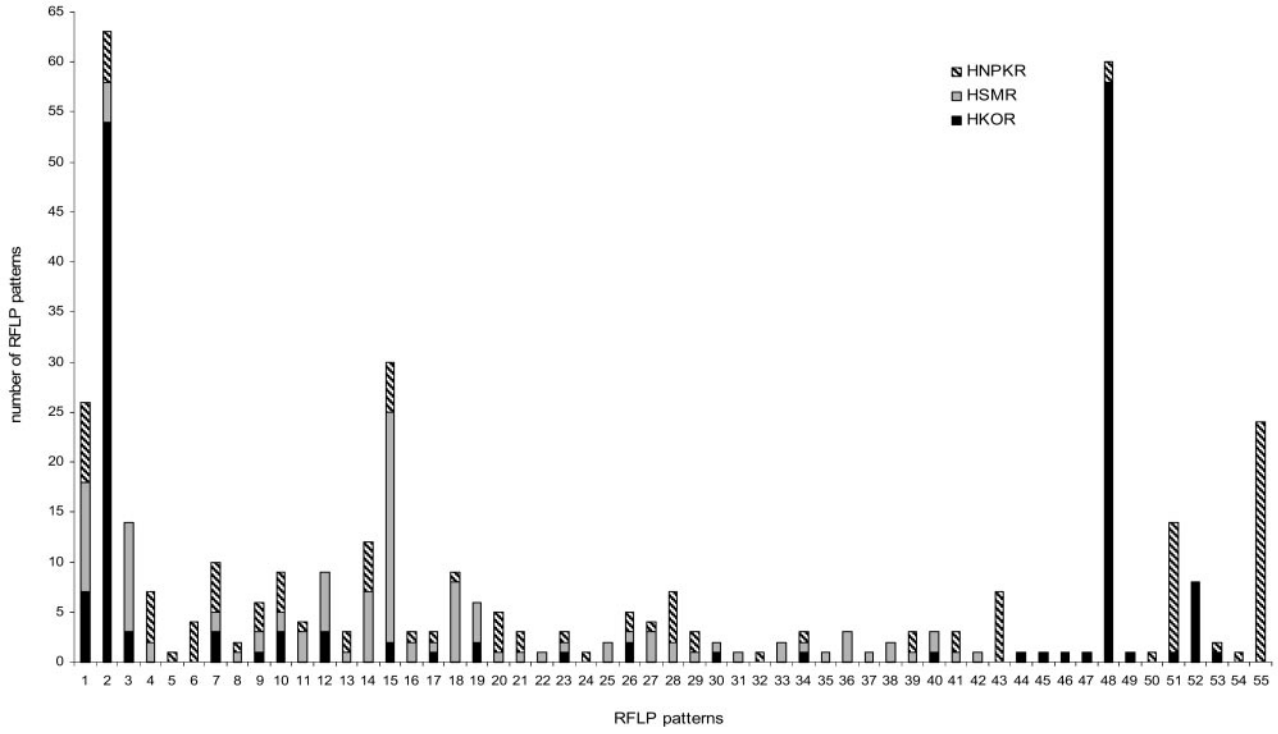

FIG. 1. Distribution of RFLP patterns of *cbbL* gene fragments from the red-like *cbbL* gene libraries of the HKO, HSM, and HNPK soil samples. *cbbL* PCR products were digested with the restriction endonuclease BbvI.

obtained from HKO soil were analyzed, and 12 clones were identified as positive for *cbbL* after restriction of the plasmids with EcoRI. For the library that was established from the HNPK soil sample, 155 clones were analyzed, but again, only 12 clones showed the correct fragment size after EcoRI restriction. Sequence analyses of selected *cbbL* clones with inserts that were shorter or longer than expected revealed that these clones were not related to *cbbL*. Due to the fact that we obtained an insufficient number of positive green-like *cbbL* clones, we did not perform RFLP analysis but sequenced all 24 clones.

Red-like *cbbL* clone libraries. The PCR products that were amplified by use of the red-like gene-specific *cbbL* primers from the three differently managed soil samples, HKO, HSM, and HNPK, were used to construct clone libraries of red-like *cbbL* genes. A total of 405 of 624 clones from the different gene libraries showed the correct fragment size. The inserts were restricted with EcoRI and screened by RFLP analysis. The number and abundance of restriction patterns were used as measures of *cbbL* diversity in the different soil samples. Fifty-five different patterns (designated 1 to 55) were found in the libraries, and the pattern types were not distributed evenly among the different soil clone libraries. We identified 24 different restriction patterns for 158 screened clones from the HKO gene library. A larger number of different patterns was detected in the gene libraries derived from HSM and HNPK soils, although a smaller number of clones was screened by RFLP analysis. We obtained 38 different patterns for 121 HSM clones and 35 different patterns for 126 HNPK clones. Thus, HKO soil contained relatively limited *cbbL* diversity compared to the HSM and HNPK soils, which showed similar high levels of diversity. The distribution of the different RFLP patterns from the three *cbbL* libraries is shown in Fig. 1. RFLP types 2 (34%) and 48 (37%) dominated among the HKO clones, while RFLP type 15 accounted for the majority of the HSM clones (19%) and RFLP type 55 accounted for the majority of the HNPK clones (43%). Additionally, all three investigated clone libraries contained RFLP types which were less abundant and were represented by only a single clone. Several RFLP patterns were only present in one of the three established libraries. Thirty-eight clones with RFLP types that occurred more than once and eight representatives with unique RFLP patterns were selected from the three *cbbL* gene libraries and then sequenced.

TABLE 3. Diversity indices obtained for red-like *cbbL* libraries from HKO, HSM, and HNPK soil samples

Diversity index	Value for soil sample		
	HKO	HSM	HNPK
S^a	24	38	35
H^b	1.89	3.18	3.06
D^c	3.86	14.08	14.29
E^d	0.59	0.87	0.86
C^e (%)	92	86	89

[a] Species richness, *S,* was calculated as the total number of different RFLP patterns in a library.
[b] Shannon-Weaver diversity index (28).
[c] Simpson's diversity index (31).
[d] Evenness (28) of the population.
[e] Library coverage (6).

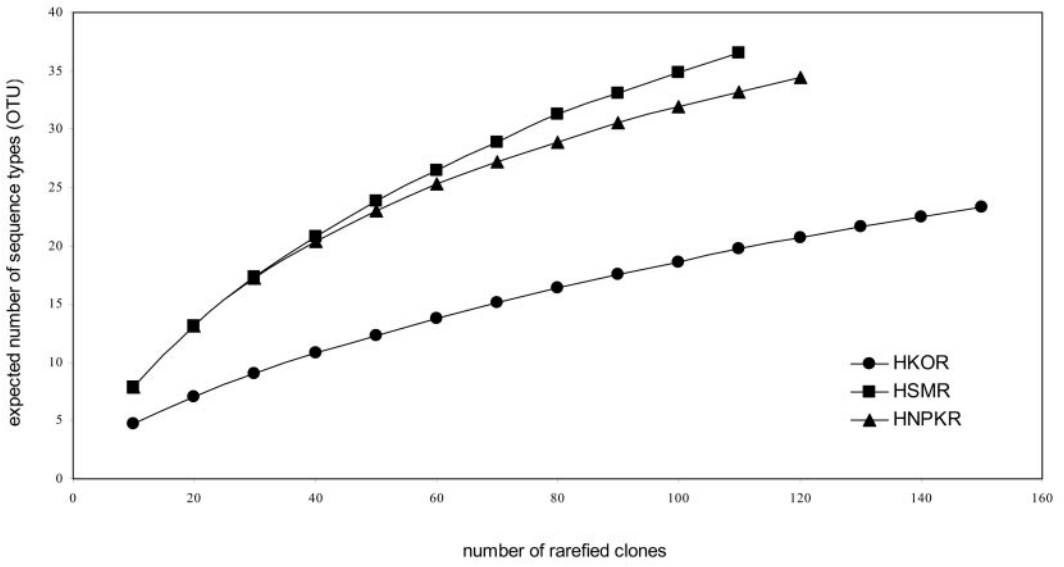

FIG. 2. Rarefaction curves for the expected number of OTUs represented by different RFLP patterns from the red-like *cbbL* gene libraries.

Diversity indices of red-like *cbbL* clone libraries. In order to extrapolate similarities or differences among the red-like *cbbL* clone libraries, we performed statistical analyses. Biodiversity analyses were possible because the clone libraries were created under almost identical conditions. Table 3 shows the diversity indices that were used to compare the gene libraries. The diversity indices of the HSM and HNPK libraries were high and similar to each other, in contrast to the indices obtained for the HKO clone library. The Shannon-Weaver, Simpson's, and evenness values indicated that the diversity of *cbbL* sequences from HSM and HNPK soils differed drastically from that of sequences obtained from HKO soil. In addition, rarefaction analysis was performed on different RFLP patterns from each red-like *cbbL* gene library as representations of OTUs, with full coverage of a library expected to give a plateau-shaped curve (Fig. 2). Rarefaction analysis confirmed the results obtained with the diversity indices. Rarefaction curves of HSM and HNPK soils represented nonasymptotic curves. The high levels of diversity of the HSM and HNPK clone libraries were also reflected in the larger numbers of different RFLP types. An underestimation of species diversity from HSM and HNPK soils is expected, as the coverage of the libraries was estimated to be 86 or 89%, respectively (Table 3). The coverage of the HKO library was higher (92%) than that of the other two soil *cbbL* libraries.

Phylogenetic analysis based on *cbbL* sequence data. (i) Green-like *cbbL* sequences from soil. Sequences were designated beginning with the soil sample name, with an added "G" for sequences from the green-like library or "R" for sequences from the red-like library, followed by the clone number in the library. Comparisons with the National Center for Biotechnology Information database by BLAST searches revealed that all sequences were clearly related to known *cbbL* sequences. A total of 24 partial green-like *cbbL* gene sequences (ca. 1,100 bp) were identified, including 12 from HKO soil and 12 from HNPK soil. The sequence similarities from pairwise comparisons of all available bacterial green-like *cbbL* sequences, including the soil clones, ranged from 60.7 to 100%. The similarity values for all 24 *cbbL* clones from the Halle soils were 90.7 to 100% and formed two subclusters (Fig. 3). The GI cluster includes sequences from the HKO soil, and the GII cluster contains sequences from the HNPK soil only. The sequence HNPKG75 is the most distantly related green-like *cbbL* clone and exhibits a sequence similarity value of 91.7% for the closest related soil clone, HKOG13. A phylogenetic tree for green-like *cbbL* nucleotide sequences (Fig. 3) shows the highest degree of relatedness for the soil clones and the sequences from *N. vulgaris* and *N. winogradskyi* (97 to 99%).

(ii) Red-like *cbbL* sequences from soil. A total of 46 red-like *cbbL* soil clones from the three differently managed soil samples, HKO, HSM, and HNPK, were analyzed. In contrast to the green-like *cbbL* sequences from Halle soil, the red-like *cbbL* sequences were much more diverse, with similarities ranging from 57.8 to 100%. The soil clone sequences were distributed all over the red-like *cbbL* cluster (Fig. 4). An influence of soil management on the distribution of red-like *cbbL* sequences was not found. Soil clones that showed the same RFLP patterns also had the same *cbbL* sequences, which is represented by a nucleotide similarity value of 100%. Most red-like *cbbL* clone sequences obtained from soil were only distantly related to already published red-like *cbbL* sequences. Moreover, we observed the formation of three new monophyletic clusters, namely, RI, RII, and RIII. The RI and RIII clusters contained only environmental *cbbL* sequences from the investigated agricultural soils.

(iii) Red-like *cbbL* sequences of bacterial strains. In order to extend the data set of red-like *cbbL* gene sequences, we examined collection cultures for the presence of *cbbL* genes by using the same primer set as that used for soil samples. The *cbbL* sequences amplified from *X. agilis* and *X. autotrophicus* grouped with already known sequences from *Xanthobacter* sp. strain COX and *Xanthobacter flavus* and showed high similarity values for these sequences (>90%). The *cbbL* sequences of *S. fredii* and *S. terangae* clustered with the *cbbL* sequence of *S. meliloti.* The *O. anthropi cbbL* sequence completed this

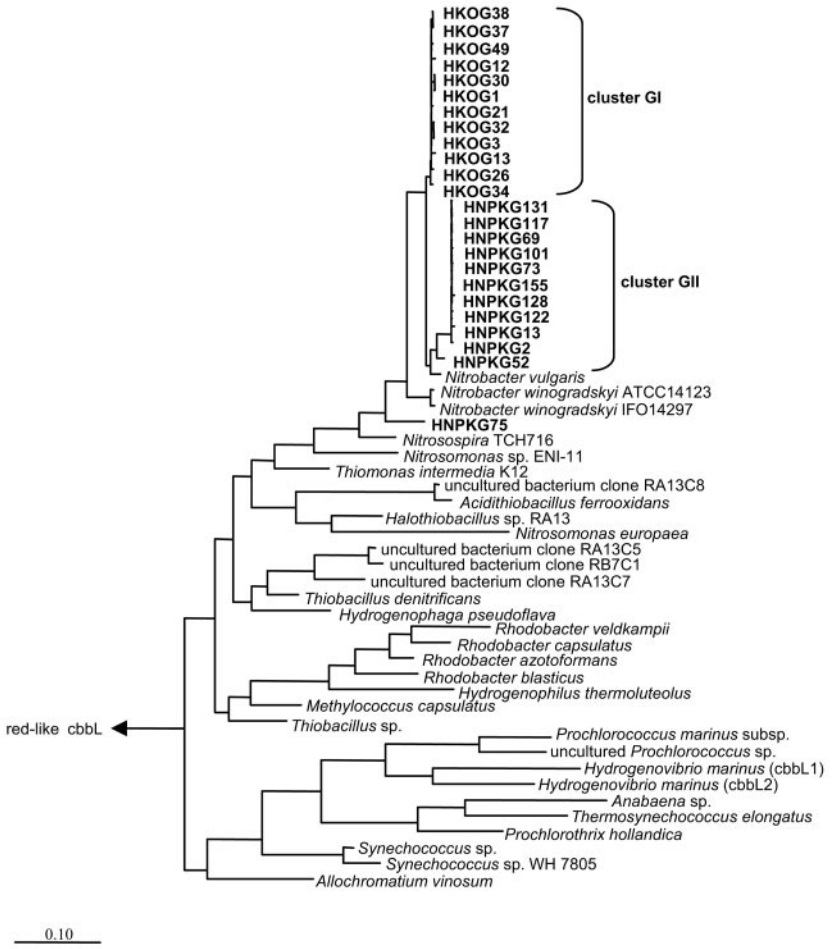

FIG. 3. Phylogenetic analysis of green-like *cbbL* genes. A consensus tree was constructed by distance (neighbor-joining), maximum parsimony, and maximum likelihood methods. The red-like *cbbL* sequence of *Ralstonia eutropha* was used as an outgroup for tree calculations. The bar indicates 10% estimated sequence divergence. Clones obtained from Halle soil samples were designated HKO or HNPK, followed by their number in the clone library. These sequences are shown in bold.

Sinorhizobium cluster, with a very high similarity value of 99.3% for *S. fredii*. Surprisingly, the *cbbL* sequences of *Azospirillum lipoferum* and *R. leguminosarum* bv. Trifolii showed low similarity values for other known red-like *cbbL* sequences. The most closely related sequence of *Azospirillum lipoferum* was that from *Bradyrhizobium* sp. strain CPP, with a similarity value of 86.3%. *R. leguminosarum* bv. Trifolii had the highest nucleotide similarity to the *cbbL* sequence of the environmental clone F38 (81.9%). Interestingly, the red-like *cbbL* clone HNPKR7 from the RII cluster showed 100% sequence similarity to the *cbbL* sequence of *R. leguminosarum* bv. Trifolii.

To further fill the gaps of red-like *cbbL* sequences, especially in the three new clusters, RI, RII, and RIII, we isolated bacteria from the HNPK soil sample by using *Rhizobium* medium. From a total of 64 isolates, 13 contained detectable *cbbL* sequences. The *cbbL* sequences of the isolates were designated with an "R," followed by the isolate number and a "c" to designate the *cbbL* gene. The nucleotide similarities of the *cbbL* sequences retrieved from the isolated bacteria ranged from 73 to 99.6%. The sequences R36c and R47c grouped into the RI cluster, and the next related *cbbL* sequence was that of HKOR22, with a similarity value of 93%. R39c and R37c joined the RII cluster. The sequence R39c exhibited the highest similarity value (90.3%) for the environmental clone HSMR29. The most related *cbbL* sequence to R37c was the soil clone sequence HNPKR1 (89.3%). The sequences R43c, R40c, and R46c showed high nucleotide similarities (>99.3%) and grouped into the RIII cluster (Fig. 4). The *cbbL* sequence of the R45 isolate completed cluster RIII, with an average similarity value of 81.7% for the other sequences within this cluster. The other nucleotide similarity values within the RIII cluster ranged from 99.6 to 100%. The remaining *cbbL* sequences of the bacterial isolates were distributed singly all over the red-like *cbbL* tree. Two isolates were obtained whose *cbbL* sequences were 100% identical to those from soil clones: R46c was identical to HKOR3D and R33c was identical to HNPKR16.

(iv) Phylogenetic analysis of *cbbL*-positive bacterial isolates based on 16S rRNA gene sequences. The phylogenetic positions of bacterial isolates harboring red-like *cbbL* genes were examined by use of the 16S rRNA gene as a phylogenetic marker. The 16S rRNA gene sequences were designated with an "R," followed by the isolate number and "S." The 16S rRNA gene sequence similarity values of all isolates ranged

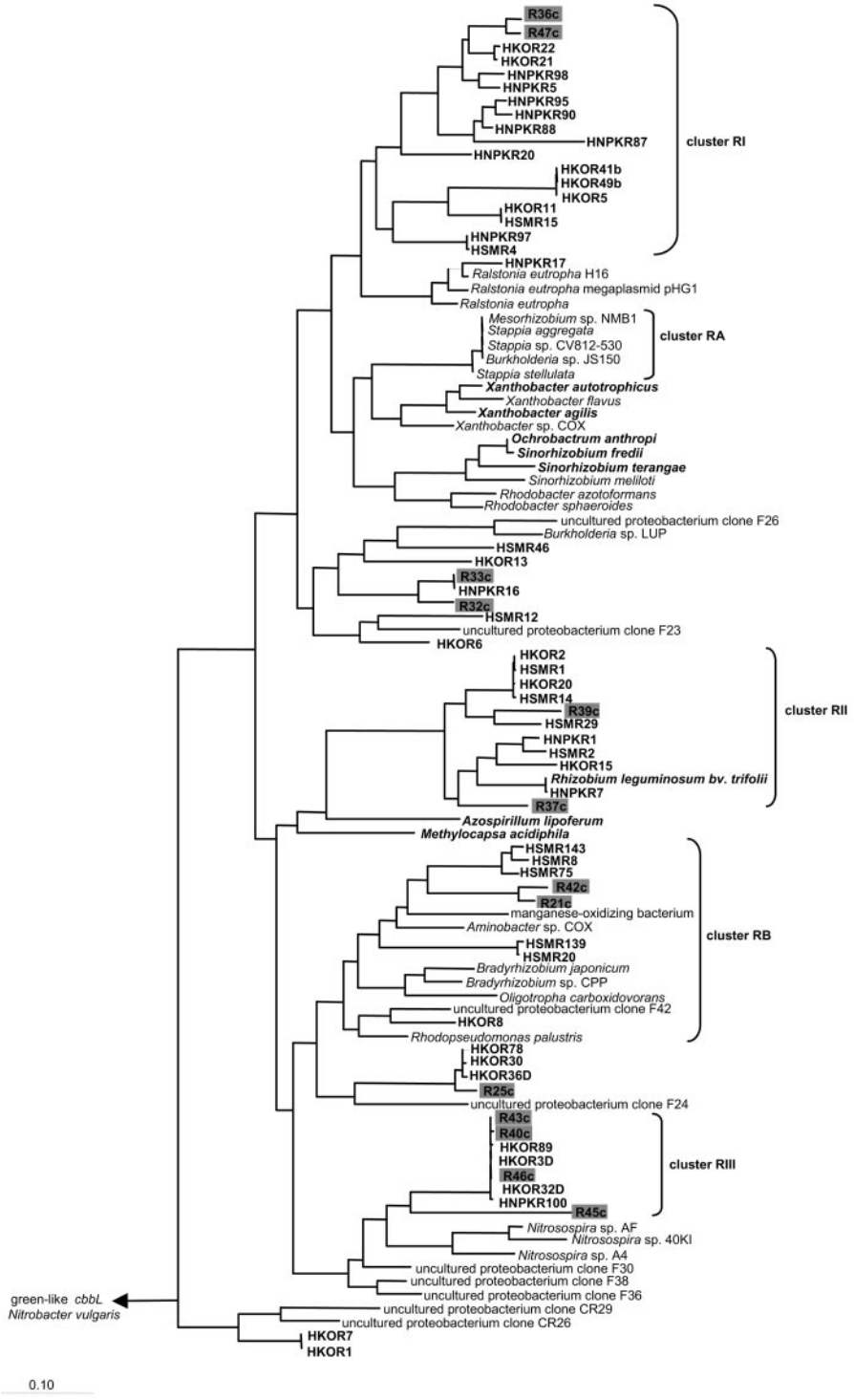

FIG. 4. Phylogenetic analysis of red-like *cbbL* genes. A consensus tree was constructed by distance (neighbor-joining), maximum parsimony, and maximum likelihood methods. The green-like *cbbL* sequence of *N. vulgaris* was used as an outgroup for tree calculations. The bar represents 0.1 changes per nucleotide or amino acid. Clones obtained from Halle soil samples were designated HKO, HSM, or HNPK, followed by their number in the clone library. The *cbbL* sequences amplified from bacterial strains and the environmental clone sequences are shown in bold. The *cbbL* sequences of bacterial isolates are designated with an "R" followed by a number and a "c." These sequences are shaded in gray.

from 80.2 to 99.6%. The similarity values of the 16S rRNA gene sequences of the isolates for already known sequences were remarkably high, ranging from 98 to 99.7%. The R33S sequence had a 98% similarity to the sequences of *Arthrobacter pascens* and *Arthrobacter ramosus*. Phylogenetic analysis revealed (Fig. 5) that 7 of 13 sequences grouped with *Bacillus* species sequences, with very high similarity values (>99.5%). Furthermore, five 16S rRNA gene sequences (R33S, R36S,

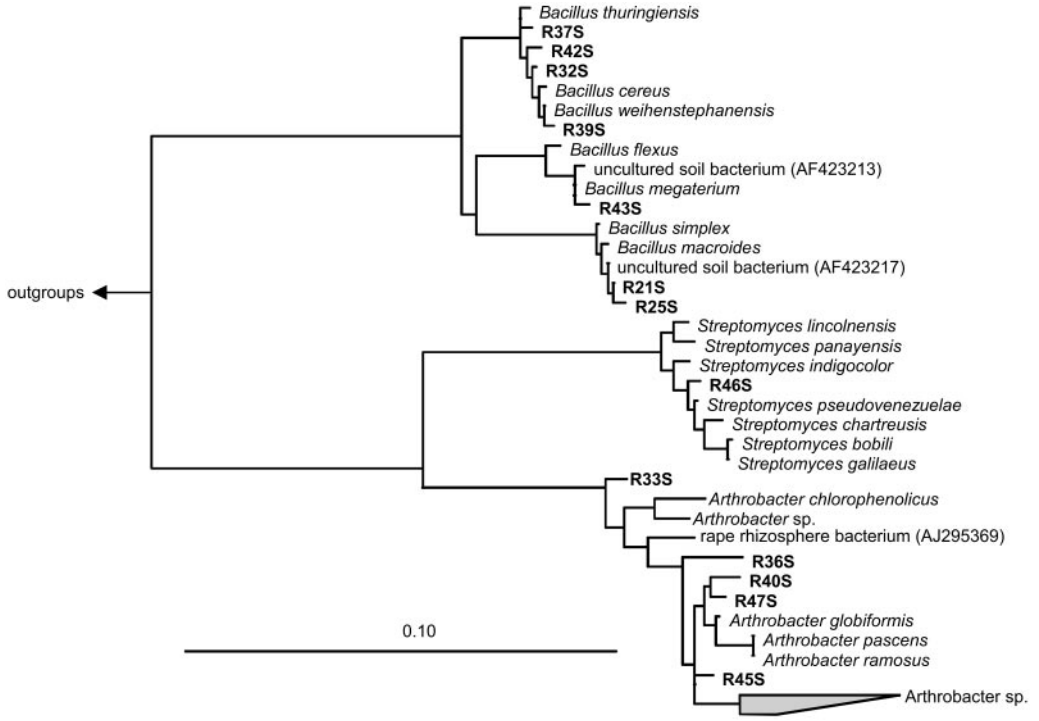

FIG. 5. Phylogenetic 16S rRNA gene sequence consensus tree reflecting the relationships of red-like *cbbL*-containing bacterial isolates. The sequences of the isolates are designated with an "R" followed by a number and an "S," and they are shown in bold. An encompassing collection of organisms representing all major lineages of the *Archaea* and *Bacteria* were used as outgroups for tree calculations. The bar indicates 10% estimated sequence divergence.

R40S, R45S, and R47S) were affiliated with a monophyletic cluster of *Arthrobacter* species. The nucleotide similarity values of the soil isolates and of cultured representatives of this group ranged from 98 to 99.5%, with the highest similarity between the sequences from isolate R47S and *Arthrobacter globiformis*. Interestingly, the 16S rRNA gene sequence of the R46 isolate was affiliated with the *Streptomyces pseudovenezuelae* sequence, with a nucleotide similarity of 99.5%.

DISCUSSION

This is the first reported investigation of the molecular diversity of RubisCO form I large-subunit *cbbL* genes in agricultural soils. Light-dependent CO_2 fixation by plants, algae, and cyanobacteria is restricted in terrestrial environments to the top few millimeters of the soil profile. In deeper soil layers, light-independent bacterial CO_2 fixation may play a hitherto underestimated important role. Only a few soil bacteria, including some heterotrophic, mostly root-associated bacteria and the chemolithoautotrophic nitrifying bacteria, have been known to harbor *cbbL* genes. We showed here that all green-like *cbbL* sequences obtained from agricultural soils by the use of our primer system were closely related to *N. vulgaris* and *N. winogradskyi*. In contrast, the red-like *cbbL* genes retrieved from three different soil samples were unexpectedly highly diverse. In fact, we found only one terrestrial red-like *cbbL* clone (HNPKR7) that was identical to a *cbbL* gene of a cultivated bacterium (*R. leguminosarum* bv. Trifolii). However, this does not necessarily imply that the environmental sequence HNPKR7 was retrieved from *R. leguminosarum* bv. Trifolii, since the *cbbL* phylogeny is incongruent with the phylogeny based on 16S rRNA genes (4). The incongruity of 16S rRNA and *cbbL* gene sequences became obvious when we compared phylogenetic affiliations based on 16S rRNA genes of the red-like *cbbL* RA cluster with red-like *cbbL* similarity values. For example, the RA cluster contains *cbbL* sequences from bacteria which belong to both α- and β-*Proteobacteria* but whose *cbbL* sequences are identical. Delwiche and Palmer (4) postulated that multiple processes are involved in the incongruity of the *cbbL* phylogeny, including horizontal gene transfer and gene duplication associated with differential gene loss. Thus, they suggested that at least four independent horizontal gene transfers are responsible for the green-like–red-like split (4). Another example is now given by the RIII cluster, which contains closely related *cbbL* sequences from *Bacillus*, *Streptomyces*, and *Arthrobacter* isolates. However, there are some surprising conservative exceptions, since the red-like *cbbL* genes of *X. agilis* and *X. autotrophicus* as well as those of *S. fredii* and *S. terangae* cluster together with already known *cbbL* sequences of phylogenetically closely related *Xanthobacter* and *Sinorhizobium* species, respectively. In these cases, there is congruency with the 16S rRNA gene phylogeny.

Unexpectedly, the isolation experiments revealed *cbbL* sequences in hitherto unknown autotrophic bacteria. The phylogenetic positions of red-like *cbbL*-containing isolates were determined by 16S rRNA gene sequence analyses. We identified bacteria belonging to the gram-positive genera *Bacillus*, *Streptomyces*, and *Arthrobacter*. A RubisCO-like protein, classified

as form IV RuBisCO, was previously detected in *B. subtilis* (17). Recently, Ashida et al. (1) clearly demonstrated that this RubisCO-like protein is involved in the methionine pathway, which had been predicted already by studies of other groups (7, 26). However, based on gene sequence data, the form IV RubisCO-like gene *ykrW* of *B. subtilis* (17) is clearly different from the form I red-like *cbbL* sequences of the gram-positive isolates shown in this study.

Examinations of the evolutionary relationships of specific functional bacterial groups by use of the 16S rRNA gene and a corresponding functional marker gene have been performed before in several instances (10, 15, 28, 35). Nitrogen-fixing bacteria can be detected by use of the *nifH* gene encoding the nitrogenase reductase (35), and methanotrophic bacteria can be detected by use of the *pmoA* gene, which encodes the α subunit of the particulate methane monooxygenase (10). The phylogenies of *nifH* and *pmoA* are (with only a few exceptions) congruent with the phylogenies based on 16S rRNA genes (10, 35). Another example of a functional gene which is used as a functional marker is the *amoA* gene (28). The evolutionary relationships of ammonia-oxidizing bacteria with the 16S rRNA gene phylogeny are similar (28). In contrast, the detection of *dsrAB*, which is used as a functional gene of sulfate-reducing bacteria, shows a partial inconsistency with the corresponding 16S rRNA gene phylogeny (15).

The discovery of the RI, RII, and RIII clusters clearly demonstrates that soils harbor an unprecedented high level of diversity among red-like *cbbL* sequences. In addition, these sequences were only distantly related to known *cbbL* sequences from public databases (Fig. 4). However, it is not possible to extrapolate from this high genetic diversity of red-like RubisCO proteins in terrestrial environments to their physiological and ecological roles because the present data are only based on *cbbL* gene diversity. However, it was previously shown by the use of $^{14}CO_2$ labeling experiments of soils that $^{14}CO_2$ is fixed to a level of 3 to 5% of the respiration rate (25). This activity was stimulated by the addition of an organic substrate and was completely abolished after soil fumigation (25). In addition, preliminary results have demonstrated that *cbbL* mRNAs can be detected in the soils studied, especially after the addition of a substrate. This points to the possible importance of mixotrophic CO_2 fixation in soils that are activated with carbon substrates. Interestingly, the diversity of red-like *cbbL* genes was increased in soils which were regularly fertilized (HNPK and HSM), although no obvious correlation could be found between the type of fertilization and the *cbbL* sequence types. We assume that fertilization favors the diversity of *cbbL*-bearing bacteria by directly or indirectly providing more available energy substrates for soil microbes.

Whether it is a general phenomenon that heterotrophic bacteria such as *Arthrobacter*, *Bacillus*, and *Streptomyces* use *cbbL* genes and RubisCO under certain ecophysiological conditions still needs to be studied in detail. Facultative chemoautotrophic or mixotrophic bacteria are capable of utilizing a wide range of substrates. Therefore, they may have an improved niche quality which would be advantageous in diverse soil habitats with rapidly changing conditions.

In contrast to the red-like sequences, the diversity of the green-like *cbbL* sequences seems rather limited in agricultural soils. Using our primer system, we detected *cbbL* sequences which were closely affiliated exclusively with the nitrite-oxidizing bacteria *N. vulgaris* and *N. winogradskyi*. The phylogenetic tree based on green-like *cbbL* sequences contains predominantly sequences of bacteria living in aquatic habitats, such as different *Cyanobacteria* and *Rhodobacter* species. These microorganisms are highly diverse and are distributed all over the green-like group (Fig. 3). Since we investigated soil samples, the low diversity of the identified green-like *cbbL* sequences, limited to the nitrifying bacterium *Nitrobacter*, can be explained. In the soil that was treated with 12 tons of manure ha^{-1} $year^{-1}$, green-like *cbbL* genes were not found. This heavy manuring resulted in a 30% increase in organic carbon in the soil (24). Nitrifiers are possibly underrepresented in this soil, with an increased heterotrophic microbial community. The content of organic carbon in the soil was not influenced much by mineral fertilization, and in the unfertilized HKO soil, organic carbon declined to the range of 10% (24). The lower content of organic carbon in HKO soil may provide limiting conditions for the diversity of red-like *cbbL* gene-carrying bacteria and may favor chemolithoautotrophic bacteria.

In conclusion, this communication revealed a huge unprecedented red-like *cbbL* diversity in agricultural soils under three different long-term soil management practices. In a related study, a very similar diversity pattern of *cbbL* genes was found in an agricultural soil of a different type (loamy cambisol from Loess at Scheyern, upper Bavaria, Germany) (I. M. Pattis, unpublished data). This new knowledge about RubisCO form I genes in soil bacteria is certainly dependent on the primer systems used and should be further supplemented by studying more terrestrial habitats or other types of RubisCO genes, such as those corresponding to form II RubisCO. Future functional studies of the newly discovered red-like *cbbL* genes are necessary to understand the ecophysiological role of *cbbL* genes in soil bacteria such as *Bacillus*, *Arthrobacter*, and *Streptomyces*. Experiments to study the activities of RubisCO proteins in these bacteria are currently being performed. Moreover, more knowledge about the in situ activities of bacteria carrying *cbbL* genes is necessary. *cbbL* mRNAs can be retrieved from soils, and the diversity of these mRNAs is expected to reveal functionally active, *cbbL*-carrying bacteria. This will eventually contribute to a better understanding of the ecological role of the autotrophy of soil bacteria for CO_2 dynamics in soils.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft in the framework of the project network "Soils as source and sink for CO_2—mechanisms and regulation of organic matter stabilisation in soils" (SPP 1090).

We thank Angelika Schulz for excellent technical support.

REFERENCES

1. **Ashida, H., Y. Saito, C. Kojima, K. Kobayashi, N. Ogasaware, and A. Yokota.** 2003. A functional link between RuBisCO-like protein of *Bacillus* and photosynthetic RuBisCO. Science **302:**286–290.
2. **Bainbridge, G., P. Madgwick, S. Parmar, R. Mitchell, M. Paul, J. Pitts, A. J. Keys, and M. A. J. Parry.** 1995. Engineering rubisco to change its catalytic properties. J. Exp. Bot. **46:**1269–1276.
3. **Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. Fitzgerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougherty, J. F. Tomb, M. D. Adams, C. I. Reich, R. Overbeek, E. F. Kirkness, K. G. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. Geoghagen, and J. C. Venter.** 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii.* Science **273:**1058–1073.

4. **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.
5. **Ellis, R. J.** 1979. The most abundant protein in the world. Trends Biochem. Sci. **4:**241–244.
6. **Good, I. J.** 1958. The population frequency of species and the estimation of the population parameters. Biometrics **40:**237–246.
7. **Grundy, F. J., and T. H. Henkin.** 2002. Biosynthesis of serine, glycine, cysteine, and methionine, p. 245–254. *In* A. L. Sonenshein, R. M. Losick, and J. A. Hoch (ed.), *Bacillus subtilis* and its relatives: from genes to cells. American Society for Microbiology, Washington, D.C.
8. **Hanson, T. E., and F. R. Tabita.** 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. USA **98:**4397–4402.
9. **Heck, K. L., G. van Belle, and D. Simberloff.** 1975. Explicit calculation of the rarefaction diversity measurement and the determination of sufficient sample size. Ecology **56:**1459–1461.
10. **Horz, H. P., M. Tchawa Yimga, and W. Liesack.** 2001. Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA*, *mmoX*, *mxaF*, and 16S rRNA and ribosomal DNA, including *pmoA*-based terminal restriction fragment length polymorphism profiling. Appl. Environ. Microbiol. **67:**4177–4185.
11. **Hurlbert, S. H.** 1971. The nonconcept of species diversity: a critique and alternative parameters. Ecology **52:**577–586.
12. **Jordan, D. B., and W. L. Ogren.** 1983. Species variation in kinetic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase. Arch. Biochem. Biophys. **227:**425–433.
13. **Juretschko, S., G. Timmermann, M. Schmid, K. H. Schleifer, A. Pommerening-Röser, H. P. Koops, and M. Wagner.** 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. **64:**3042–3051.
14. **Kellogg, E. A., and N. D. Juliano.** 1997. The structure and function of RuBisCO and their implications for systematic studies. Am. J. Bot. **84:** 413–428.
15. **Klein, M., M. Friedrich, A. J. Roger, P. Hugenholtz, S. Fishbain, H. Abicht, L. L. Blackall, D. A. Stahl, and M. Wagner.** 2001. Multiple lateral transfers of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes. J. Bacteriol. **183:**6028–6035.
16. **Klenk, H. P., R. A. Clayton, J. F. Tomb, O. White, K. E. Nelson, K. A. Ketchum, R. J. Dodson, M. Gwinn, E. K. Hickey, J. D. Peterson, D. L. Richardson, A. R. Kerlavage, D. E. Graham, N. C. Kyrpides, R. D. Fleischmann, J. Quackenbush, N. H. Lee, G. G. Sutton, S. Gill, E. F. Kirkness, B. A. Dougherty, K. McKenney, M. D. Adams, B. Loftus, J. C. Venter, et al.** 1997. The complete genome of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus.* Nature **390:**364–370.
17. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis.* Nature **390:**249–256.
18. **Kusian, B., and B. Bowien.** 1997. Organization and regulation of *cbb* CO_2 assimilation genes in autotrophic bacteria. FEMS Microbiol. Rev. **21:**135–155.
19. **Lebuhn, M., W. Achouak, M. Schloter, O. Berge, H. Meier, M. Barakat, A. Hartmann, and T. Heulin.** 2000. Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. Int. J. Syst. Evol. Microbiol. **6:**2207–2223.
20. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüssmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1–9.
21. **Malik, K. A., and H. G. Schlegel.** 1981. Chemolithoautotrophic growth of bacteria able to grow under N_2-fixing conditions. FEMS Microbiol. Lett. **11:** 63–67.
22. **McFadden, B. A., J. Torres-Ruiz, H. Daniell, and G. Sarojini.** 1986. Interaction, functional relations and evolution of large and small subunits in RuBisCO from prokaryota and eukaryota. Philos. Trans. R. Soc. Lond. Biol. Sci. **313:**347–358.
23. **Merbach, W., L. Schmidt, and L. Wittenmeyer.** 1999. Die Dauerdüngungsversuche in Halle (Saale). Teubner, Stuttgart, Germany.
24. **Merker, J.** 1956. Untersuchungen an den Ernten und Böden des Versuches "Ewiger Roggenbau" in Halle. Kühn-Arch. **70:**154–215.
25. **Miltner A., F. D. Kopinke, R. Kindler, D. Selesi, A. Hartmann, and M. Kästner.** Non-phototrophic CO_2 fixation by soil microorganisms. Plant Soil, in press.
26. **Murphy, B. A., F. J. Grundy, and T. M. Henkin.** 2002. Prediction of gene function in methylthioadenosine recycling from regulatory signals. J. Bacteriol. **184:**2314–2318.
27. **Paoli, G. C., N. S. Morgan, F. R. Tabita, and J. M. Shively.** 1995. Expression of the *cbbL*, *cbbS* and *cbbM* genes and distinct organization of the *cbb* Calvin cycle structural genes of *Rhodobacter capsulatus.* Arch. Microbiol. **164:**396–405.
28. **Purkhold, U., A. Pommerening-Röser, S. Juretschko, M. C. Schmid, H. P. Koops, and M. Wagner.** 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. **66:** 5368–5382.
29. **Shannon, C. E., and W. Weaver.** 1963. The mathematical theory of communication, p. 117. University of Illinois Press, Urbana, Ill.
30. **Shively, J. M., W. Devore, L. Stratford, L. Porter, L. Medlin, and S. E. Stevens.** 1986. Molecular evolution of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). FEMS Microbiol. Lett. **37:** 251–257.
31. **Simberloff, D.** 1978. Use of rarefaction and related methods, p. 150–165. *In* K. L. Dickson et al. (ed.), Biological data in water pollution assessment: quantitative and statistical analyses. American Society for Testing and Materials, Philadelphia, Pa.
32. **Simpson, E. H.** 1949. Measurement of diversity. Nature **163:**688.
33. **Tabita, F. R.** 1988. Molecular and cellular regulation of autotrophic carbon fixation in microorganisms. Microbiol. Rev. **52:**155–189.
34. **Uchino, Y., and A. Yokota.** 2003. "Green-like" and "red-like" RubisCO *cbbL* genes in *Rhodobacter azotoformans.* Mol. Biol. Evol. **20:**821–830.
35. **Ueda, T., Y. Suga, N. Yahiro, and T. Matsuguchi.** 1995. Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. J. Bacteriol. **177:**1414–1417.
36. **Watson, G. M., and F. R. Tabita.** 1997. Microbial ribulose-1,5-bisphosphate carboxylase/oxygenase: a molecule for phylogenetic and enzymological investigations. FEMS Microbiol. Lett. **146:**13–22.