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Identification of Unknown Carboxydovore Bacteria Dominant in Deciduous Forest Soil via Succession of Bacterial Communities, *coxL* Genotypes, and Carbon Monoxide Oxidation Activity in Soil Microcosms

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Surveys of the *coxL* gene, encoding the large subunit of the CO dehydrogenase, are used as a standard approach in ecological studies of carboxydovore bacteria scavenging atmospheric CO. Recent soil surveys unveiled that the distribution of *coxL* sequences encompassing the atypical genotype *coxL* type I group x was correlated to the CO oxidation activity. Based on phylogenetic analysis including the available *coxL* reference genome sequences, this unusual genotype was assigned to an unknown member of the *Deltaproteobacteria*, with the *coxL* sequence from *Haliangium ochraceum* being the sole and closest reference sequence. Here we seek to challenge the proposed taxonomic assignation of the *coxL* group x genotype through the monitoring of CO consumption activity and microbial community successions during the colonization of sterile soil microcosms inoculated with indigenous microorganisms. In our study, we established that the estimated population density of *Deltaproteobacteria* was too small to account for the abundance of the *coxL* group x genotype detected in soil. Furthermore, we computed a correlation network to relate 16S rRNA gene profiles with the succession of *coxL* genotypes and CO uptake activity in soil. We found that most of the *coxL* genotypes for which the colonization profile displayed covariance with CO uptake activity were related to potential carboxydovore bacteria belonging to *Actinobacteria* and *Alphaproteobacteria*. Our analysis did not provide any evidence that *coxL* group x genotypes belonged to *Deltaproteobacteria*. Considering the colonization profile of CO-oxidizing bacteria and the theoretical energy yield of measured CO oxidation rates in soil microcosms, we propose that unknown carboxydovore bacteria harboring the atypical *coxL* group x genotype are mixotrophic *K*-strategists.

Carbon monoxide (CO) is a trace gas in the atmosphere, with a typical mixing ratio of 60 to 150 ppb by volume (ppbv) (1). Combustion of fossil fuels and biomass burning are the main sources of this gas (2). The hydroxyl radicals (OH˙) are responsible for 85% of the global sink of CO, while specialized aerobic microorganisms in the soil consume the remaining 15% (3, 4). These CO-oxidizing bacteria represent the most uncertain term for the budget of atmospheric CO. The diversity and ecophysiology of this ubiquitous functional group present in the environment are poorly documented and impair the fate prediction of the biological sink and the atmospheric burden CO in response to global change.

CO-oxidizing bacteria possess a CO dehydrogenase enzyme (CODH) catalyzing the following reaction: $CO + H_2O \rightarrow CO_2 + 2H^- + 2e^+$. The enzyme is composed of a dimer of heterotrimers with CoxS, CoxM, and CoxL as small, medium, and large subunits, respectively (5). The CoxL subunit contains the active site of the enzyme and is used as a molecular marker to assess CO-oxidizer diversity in the environment (6). Furthermore, the *coxL* gene sequences encompass two different phylogenetic groups: the type I group that has been genetically and biochemically characterized and the putative type II group. There is compelling evidence suggesting that type II CODH is not functional or uses another substrate than CO (7, 8), and thus *coxL* sequences encompassing this group are not considered in an environmental survey of CO-oxidizing bacteria. CO-oxidizing bacteria harboring *coxL* type I comprise two different physiological groups. They are composed of the carboxydotrophic bacteria, displaying low affinity for CO and the ability to grow using CO as the sole carbon and energy source, and the carboxydovore bacteria, displaying high affinity for CO, enabling the capacity to scavenge the trace amounts present in the atmosphere (9). Only a few carboxydovore bacteria have been extensively characterized, encompassing *Alphaproteobacteria* (*Bradyrhizobium* spp., *Stappia* spp., *Aminobacter* sp., and *Ruegeria pomeroy* DSS-3) (10–12), *Betaproteobacteria* (*Burkholderia* spp.) (13), *Gammaproteobacteria* (*Alkalilimnicola ehrlichii* and *Streptomonas* sp. strain LUP) (10, 14), *Actinobacteria* (*Mycobacterium* spp.) (10), and *Chloroflexi* (*Ktedonobacteria*) (15). However, the environmental factors determining their activity and distribution remain elusive (9).

Previously published environmental surveys of *coxL* sequences have been done in volcanic deposits (6, 16, 17), hot springs (18), and aquatic environments (19). Even though forest ecosystems exert a dominant role in the global sink of atmospheric CO (20), the carboxydovore bacteria thriving in forest soil have received

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little attention. Recently, a soil survey of *coxL* type I sequences in deciduous forest have led to the identification of a new *coxL* group, referred to as *coxL* group x in this paper, for which the distribution was correlated with CO soil uptake activity. A phylogenetic analysis identified the reference *coxL* sequence from *Haliangium ochraceum* as the closest relative of the *coxL* group x genotype suggesting the presence of unknown CO-oxidizing *Deltaproteobacteria* in soil (21). However, this assumption is highly uncertain because of the potential lateral transfer of the *coxL* gene and the relatively low identity score (less than 75%) between *coxL* group x sequences retrieved from soil and the reference *coxL* sequence in *H. ochraceum*. To address the problematic nature of this assumption, our study aimed to challenge the hypothetical affiliation of the *coxL* group x genotype with *Deltaproteobacteria*. The succession of bacterial communities, *coxL* genotypes, and the CO oxidation activity were monitored in sterile deciduous forest soil microcosms inoculated with the indigenous microorganisms. Also, we verified the hypothesis that the maturation of the CO oxidation activity would be related to the concomitant emergence of the *coxL* group x genotype and 16S rRNA gene sequences from *Deltaproteobacteria*.

MATERIALS AND METHODS

Soil sample and incubation. The soil sample was collected from a deciduous forest located about 40 km from Montreal on the south shore of the St. Lawrence River (45°67′N, 73°32′W). Soil texture was determined by the hydrometer method, and particle size distribution assigned soil samples to the silt loam textural class (22). The same site was visited to investigate the impact of land use change, soil nutrients, and physicochemical properties on CO-oxidizing bacteria (21). The soil sample was homogenized (2-mm-pore sieve) and used for the preparation of the colonization substrates and inocula. For the colonization substrate, soil was sterilized two times, separated by a 24-h interval. Sterilization was done in 500-ml Wheaton glass bottles closed with a foam cap. Three bottles were prepared containing 72 g of soil. After the sterilization procedure, the bottles were kept on the laboratory bench with a foam cap for a 5-day period in order to evacuate CO produced during autoclaving. Sterile soil microcosms were inoculated with 8 g of homogenized nonsterile soil (i.e., indigenous soil microorganisms), corresponding to a 10% (vol/vol) inoculum. Three bottles were treated in the same manner with 80 g of sterile soil without inoculation for the controls. The sterile foam cap was kept during the incubation at 20°C to allow gaseous exchanges in the microcosms. After 0, 4, 9, 12, 15, 22, 36, and 55 incubation days, subsamples of 5.5 g were collected in the six microcosms for DNA extraction (0.5 g), and moisture content (5 g) was determined using the standard gravimetric method. Soil water content was maintained at approximately 35% during the incubation period. This water level was selected to avoid diffusion limitation of the CO uptake activity, while supporting bacterial metabolism and growth (23, 24). Soil samples dedicated to DNA extraction were frozen in a 2-ml microtube containing 700 μl TPM buffer (50 mM Tris-HCl [pH 7], 1.7% polyvinylpolypyrrolidone, 20 mM $MgCl_2$), 35 μl 20% SDS, and 0.5 g glass beads (0.1-mm diameter).

CO oxidation rate in soil. Before measurement of CO oxidation activity, the foam cap was replaced with a butyl septum cap. A CO gas mixture (508 ± 10 ppm by volume [ppmv] CO [GTS-Welco, PA, USA]) was injected to get an approximately 4-ppmv initial concentration in the static headspace. Headspace samples (10 ml) were collected with a Pressure Lok gastight glass syringe (VICI Precision Sampling, Inc., Baton Rouge, LA, USA) and injected in a gas chromatograph equipped with a reduction gas detector (ta3000R; Ametek Process Instruments, DE, USA). The first-order oxidation rates were calculated by integrating the CO mole fraction time series measured over a 2-h period, using at least four concentration points for data integration. After each oxidation rate measurement, a foam cap was put back onto the bottles for incubation. Reproducibility of the CO analyses was assessed before each set of measurements by repeated analyses of certified CO standard gas (2.05 ± 0.10 ppmv [GTS-Welco, PA, USA]), and standard deviations were lower than 5%.

DNA extraction and qPCR assays. Genomic DNA was extracted using a combination of chemical and mechanical cell lysis procedures (25). DNA was precipitated with 2 volumes 96% ethanol, and a polyvinylpolypyrrolidone spin column was used for final purification (26). Purified DNA extracts were eluted in 200 μl nuclease-free water. Purified DNA was used for quantitative PCR (qPCR) of bacterial 16S rRNA gene, *coxL* type I, and *coxL* type I *Deltaproteobacteria* (represented as *coxL* group x in this article) using previously described procedures (21). The reactions were performed using 5 μl of diluted genomic DNA (1:50).

***coxL* genotyping.** Genomic DNA extracted after 4, 22, 36, and 55 incubation days was used for *coxL* pyrosequencing. PCR amplification of *coxL* genes using the universal assay (21), library preparation, and 454 pyrosequencing were performed at McGill University and Génome Québec Innovation Centre. A total of 45,000 raw sequences were obtained from the 12 libraries. The sequences were analyzed using the software mothur (27). Sequences with a quality score ("qaverage") lower than 25, with more than 8 consecutive homopolymers, and with ambiguous bases were all removed. The average length of sequences was 336 bases. Sequences were aligned against a *coxL* sequences database previously built (21). An arbitrary identity cutoff of 0.04 was used for the clusterization of nucleotide sequences into operational taxonomic units (OTU) representing *coxL* sequences belonging to the same bacterial species (21). Libraries were standardized to the sequencing effort of the smallest *coxL* gene library (i.e., 641 sequences) to avoid biases in comparative analyses introduced by the sampling depth. At this stage, hypothetical *coxL* type II OTU identified by the canonical amino acid signature of their active site (AYR GAGR) were removed from the data set. The resulting OTU table was used for rarefaction and diversity analyses. OTU with less than 7 sequences (0.1% of total sequences) were eliminated, and 136 OTU were left for phylogenetic and correlation network analysis. Nucleic acid sequences were imported in the software MEGA (28) for phylogenetic analysis using the reference database and procedure previously described (21) to which sequences were added.

Bacterial 16S rRNA gene profiling. Genomic DNAs extracted after 4, 22, 36, and 55 incubation days were used for 16S rRNA gene profiling. PCR amplification of the V6 to V8 regions of 16S rRNA, library preparation, and Illumina MiSeq 250-bp paired-end sequencing reactions were performed by the technical staff of McGill University and Génome Québec Innovation Centre. The two paired-end reads (forward and reverse) were merged using Flash (29) with a minimum overlap length between the two reads of 20 bases and a maximum overlap of 250 bases. The maximum allowed ratio between the number of mismatched base pairs and the overlap length was set to 0.3. Reads were truncated to a uniform length of 420, corresponding to the length of more than 97.5% of the total reads. Reads with a low-quality score were removed using a maximum expected error value of 2.0 (30). The remaining reads (4,058,447) were processed using the software QIIME version 1.8.0 (31). Reads were dereplicated and then sorted by abundance. Unique reads (1,214,627) were then clustered at 97% identity using USEARCH (30) as the OTU picking method, with a minimum of two sequences for the OTU to be considered. The resulting OTU were given an extra check for chimeras using the Gold reference database (http://drive5.com/uchime/uchime_download.html), and those that were flagged as chimeric (10,339) were removed. The remaining 2,060 nonchimeric OTU were assigned using the "assign taxonomy" command (QIIME) run against the Greengenes database (version gg_13_8) (32). Reads were rarefied to the sequencing effort of the smallest 16S rRNA gene library (206,201 reads) to avoid biases in comparative analysis introduced by the sampling depth, giving a final number of 613 OTU.

Statistical analysis. Statistical analyses were all performed using the software R (33). The package "Hmisc" was used to measure Spearman correlation between *coxL*/16S rRNA gene ratio estimated by qPCR and

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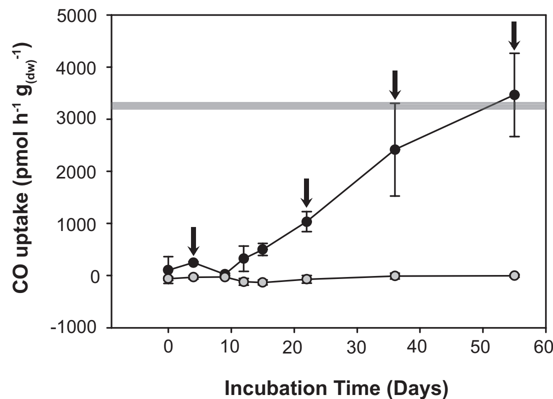

FIG 1 Time series of the CO oxidation activity measured in inoculated (black dots) and sterile (gray dots) soil microcosms. Averages are presented with standard deviations derived from three independent replicates. The gray line represents the CO oxidation rate measured in native soil. The black arrows indicate selected soil samples for high-throughput sequencing of PCR-amplified *coxL* and 16S rRNA genes. dw, dry weight.

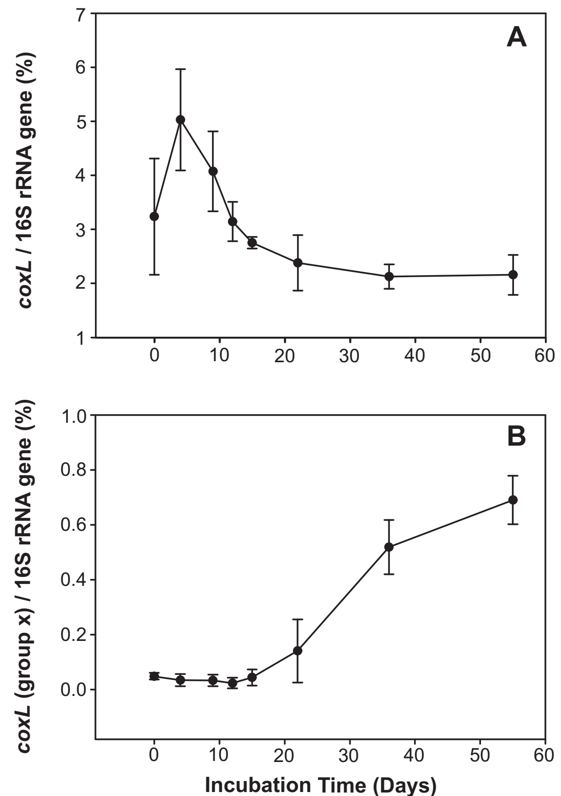

FIG 2 Time series of the relative abundance of (A) *coxL* and (B) *coxL* group x genotypes in soil microcosms as estimated by qPCR.

soil CO uptake (34). The package "Vegan" was used to compute α diversity indexes and β diversity with homogeneity multivariate dispersion (35, 36). The package Weighted Gene Co-Expression Network Analysis (WGCNA) was used to identify modules of highly correlated 16S rRNA OTU (37). The data matrix used for this analysis was expressed in 16S rRNA gene copies per gram of soil dry weight. For this purpose, the relative frequency of individual 16S rRNA gene OTU as determined through sequencing was multiplied by the absolute copy number of the 16S rRNA gene as determined by qPCR. A soft power of 12 was used to raise the Spearman correlation matrix into an adjacency matrix. This matrix was then converted into a topological overlap (TO) matrix by the TOM similarity algorithm, and genes were hierarchically clustered based on TO similarity. Modules comprising less than 10 OTU were not considered, and modules having an eigengene dissimilarity of $<$0.45 were merged. Spearman correlations were computed between the modules eigengene and CO uptake rate and the absolute abundance of *coxL* OTU. The absolute abundance of *coxL* OTU (expressed in copies per gram of soil dry weight) was computed by multiplying the relative abundance of individual OTU obtained from pyrosequencing profiles by the absolute abundance of *coxL* genes determined by qPCR.

Nucleotide sequence accession number. Raw sequences have been deposited in the Sequence Read Archive of the National Center for Biotechnology Information under BioProject no. PRJNA299500.

RESULTS

Maturation of soil CO uptake activity. The maturation of CO uptake activity was detected after a lag phase of 10 days (Fig. 1). We observed that the activity then increased continuously, reaching 3,466 ± 800 pmol g dry weight^{-1} h^{-1} after 55 days. This activity is comparable to the oxidation rate of 3,243 pmol g dry weight^{-1} h^{-1} measured in the native soil (21), indicating that activity was restored at the end of the incubation period. Furthermore, we found that no significant CO uptake activity was observed in the control experiment comprising noninoculated sterile soil (Fig. 1).

Abundance of bacterial 16S rRNA and the *coxL* gene. Using qPCR, we determined that the abundance of 16S rRNA gene increased from $(3.3 \pm 0.5) \times 10^9$ copies g dry weight $^{-1}$ at the beginning of the experiment to $(4.4 \pm 1.4) \times 10^{10}$ copies g dry weight $^{-1}$ after 4 days of incubation (see Fig. S1 in the supplemental material). No significant increase was observed between 22 and 55 incubation days, with $(2.6 \pm 0.5) \times 10^{11}$ copies g dry weight^{-1} at the end of the incubation period. Quantification of the *coxL* gene followed the same trend as the 16S rRNA gene, while *coxL* group x increased exponentially after a lag phase of 15 days (see Fig. S1). The relative abundance of *coxL* genotypes in soil was estimated by computing the *coxL*/16S rRNA gene ratio (Fig. 2). We established that the relative abundance of *coxL* increased by 5.0% ± 0.94% after 4 days and then decreased exponentially, reaching 2.2% ± 0.37% at the end of the incubation. On the other hand, the relative abundance of *coxL* group x increased exponentially from 0.05% ± 0.01% at day 15 to 0.7% ± 0.09% after 55 days of incubation. The relative abundance of *coxL* group x was positively correlated with CO uptake rate (Spearman, $P < 0.001$), while the abundance of neither the *coxL* gene nor 16S rRNA gene showed a significant relationship to CO oxidation activity.

Bacterial 16S rRNA genotypes. The sequencing effort was sufficient to cover most of the diversity of 16S rRNA genes, as assessed by rarefaction curves that reached a saturation plateau (data not shown). In total, 613 OTU were identified. Interestingly, the incubation of soil microcosms resulted in an overall decrease in species richness and species evenness (Table 1), indicating that a fraction of the bacteria present in the native soil inoculum demonstrated the ability to colonize sterilized soil with some disproportionate single-species contribution to diversity. The β diversity analysis showed that the structure of microbial communities was significantly more heterogeneous at the beginning of the incubation than observed in the last three subsamples, indicating reproducible enrichment of the subset of the initial bacterial commu-

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TABLE 1 Specific richness and evenness of *coxL* and 16S rRNA gene OTU in soil microcosms

Incubation time (day)	Diversity by index:			
	16S rRNA gene		*coxL*	
	Shannon index	Simpson index	Shannon index	Simpson index
4	4.57 ± 0.10	0.980 ± 0.001	4.43 ± 0.14	0.978 ± 0.004
22	3.45 ± 0.08	0.920 ± 0.007	4.07 ± 0.21	0.953 ± 0.004
36	3.66 ± 0.04	0.930 ± 0.006	3.87 ± 0.23	0.924 ± 0.018
55	3.84 ± 0.12	0.937 ± 0.006	3.91 ± 0.23	0.930 ± 0.018

nity in replicated microcosms (see Fig. S2A in the supplemental material). At the end of the incubation period, we found that soil microbial communities were dominated by the phyla *Actinobacteria* (65% ± 4%), *Acidobacteria* (17% ± 1%), and *Proteobacteria* (16% ± 3%). *Proteobacteria* were dominated by *Alphaproteobacteria* (16% ± 2.6%), followed by *Betaproteobacteria* (0.44% ± 0.43%), *Gammaproteobacteria* (0.12% ± 0.12%), and *Deltaproteobacteria* (0.008% ± 0.002%). Considering the abundance of the 16S rRNA gene determined by qPCR and assuming three rRNA gene copies per genome (38), we evaluated that the population density of *Deltaproteobacteria* reached 7.0×10^4 cell g dry weight^{-1} in the soil microcosm. This is well below the absolute abundance of *coxL* group x sequences determined by qPCR (1.8×10^9 copies g dry weight^{-1}).

***coxL* genotypes.** Rarefaction analysis showed that the sequencing effort was sufficient to cover *coxL* diversity (data not shown), with 136 OTU identified. As observed in the 16S rRNA gene profile, *coxL* richness and evenness decreased during soil colonization (Table 1). Homogenization of *coxL* genotype profiles in replicated microcosms was slower than that in 16S rRNA genes, and they exhibited more heterogeneity in soil subsamples collected after 4 and 22 days than was observed in the replicated microcosms sampled at days 36 and 55 (see Fig. S2B in the supplemental material). The *coxL* OTU were assigned to one of the three reference genotypes in a phylogenetic analysis (Fig. 3). At the end of the incubation, 53% ± 3% of *coxL* sequences were assigned to *coxL* group x, while *Actinobacteria* and *Alphaproteobacteria* represented 14% ± 3% and 31% ± 3% of retrieved *coxL* sequences. We noticed that few OTU dominated the three genotypes. One OTU closely related to *Bradyrhizobium japonicum* USDA (*Alphaproteobacteria* [OTU 1]) represented 70% of total sequences encompassing *Alphaproteobacteria* genotype and 18% of total *coxL* sequences (see Fig. S3A in the supplemental material). The most abundant OTU belonging to *Actinobacteria* was closely related to *Arthrobacter* sp. strain FB24 (OTU 4) and represented 24% of the sequences comprising this genotype (see Fig. S3B). OTU 2 and OTU 3 represented 21 and 11% of total sequences encompassing the *coxL* group x genotype, respectively (see Fig. S3C). The only reference bacterium present in the cluster *coxL* group x defined by the phylogenetic analysis is *H. ochraceum*. The percentage of identity between this reference sequence and the closest OTU was 80%. All of the other OTU assigned to *coxL* group x were more distantly related to *H. ochraceum*, with an identity score of between 46 and 59%.

Correlation network analysis. The CO oxidation activity, *coxL* genotypes, and 16S rRNA gene profile databases were combined to correlate the soil genetic profile with the CO oxidation activity and to test the hypothesis that *coxL* group x sequences positively correlated to activity belonged to *Deltaproteobacteria*. For this purpose, a correlation network was first constructed using the time series of 16S rRNA profiles. This analysis led to a classification of the OTU into five modules according to the covariance of their soil colonization profile (Fig. 4). The first module (module 1) was dominated by OTU encompassing *Actinobacteria* (62%), *Acidobacteria* (22%), and *Proteobacteria* (15%). The second module (module 2) was mostly composed of *Firmicutes* OTU (90%). Module 3 was the most taxonomically diverse, comprising 12 different phyla. Module 4 was mainly represented by OTU affiliated with *Actinobacteria* (60%), *Proteobacteria* (26%), and *Actinobacteria* (9%). Finally, module 5 was composed mostly of *Proteobacteria* (35%), *Actinobacteria* (26%), and *Chlamydia* (16%). Correlation of modules' eigengenes with the CO oxidation rates measured during the incubation of soil microcosms unveiled that the relative abundance of OTU encompassing module 1 was significantly correlated to the maturation of CO uptake activity in soil microcosms (Spearman, $P < 0.001$). Thus, the OTU constituting module 1 were from presumptive carboxydovore bacteria and other bacteria displaying a similar colonization profile but incapable of CO oxidation activity.

The 16S rRNA gene OTU assigned as *Acidobacteriaceae*, *Actinospicaceae*, *Microbacteriaceae*, *Bradyrhizobiaceae*, and *Acetobacteraceae* dominated the composition of module 1 with 22, 36, 21, 7, and 3% of total sequences in the module, respectively (Table 2). Most of the taxonomic groups (family level) comprising module 1 are represented by at least a reference genome or bacterium possessing *coxL* sequence (Table 2). Even though no *coxL* genes were found in the reference genomes from *Actinospicaceae*, *Microbacteriaceae*, and *Acetobacteraceae*, the colonization profile of OTU assigned to these taxonomic groups was significantly correlated with CO oxidation activity (Table 2).

Correlation of modules' eigengenes with time series of *coxL* genotypes led to the identification of 42 *coxL* OTU whose colonization profiles were significantly correlated with those of 16S rRNA gene OTU comprising module 1 (Fig. 5). Among these OTU, 27 encompassed *coxL* group x, 5 belonged to *Actinobacteria*, 8 comprised *Alphaproteobacteria*, and 2 were outside these groups and affiliated with a *coxL* sequence from *Deinococcus-Thermus*. Convergence of the taxonomic affiliation and the relative abundance of some rRNA gene OTU in module 1 and *coxL* OTU (i.e., *Bradyrhizobium* spp.) supported the phylogenetic analysis of *coxL* genes (see Table S1 in the supplemental material). No members of the *Deltaproteobacteria* were present in module 1, impairing the assignation of *coxL* group x genotype to this taxonomic group. At the end of the incubation, the absolute abundance of the 27 *coxL* group x OTU correlated with the CO uptake rate was $(2.4 \pm 0.34) \times 10^9$ bacteria g dry weight^{-1}, as

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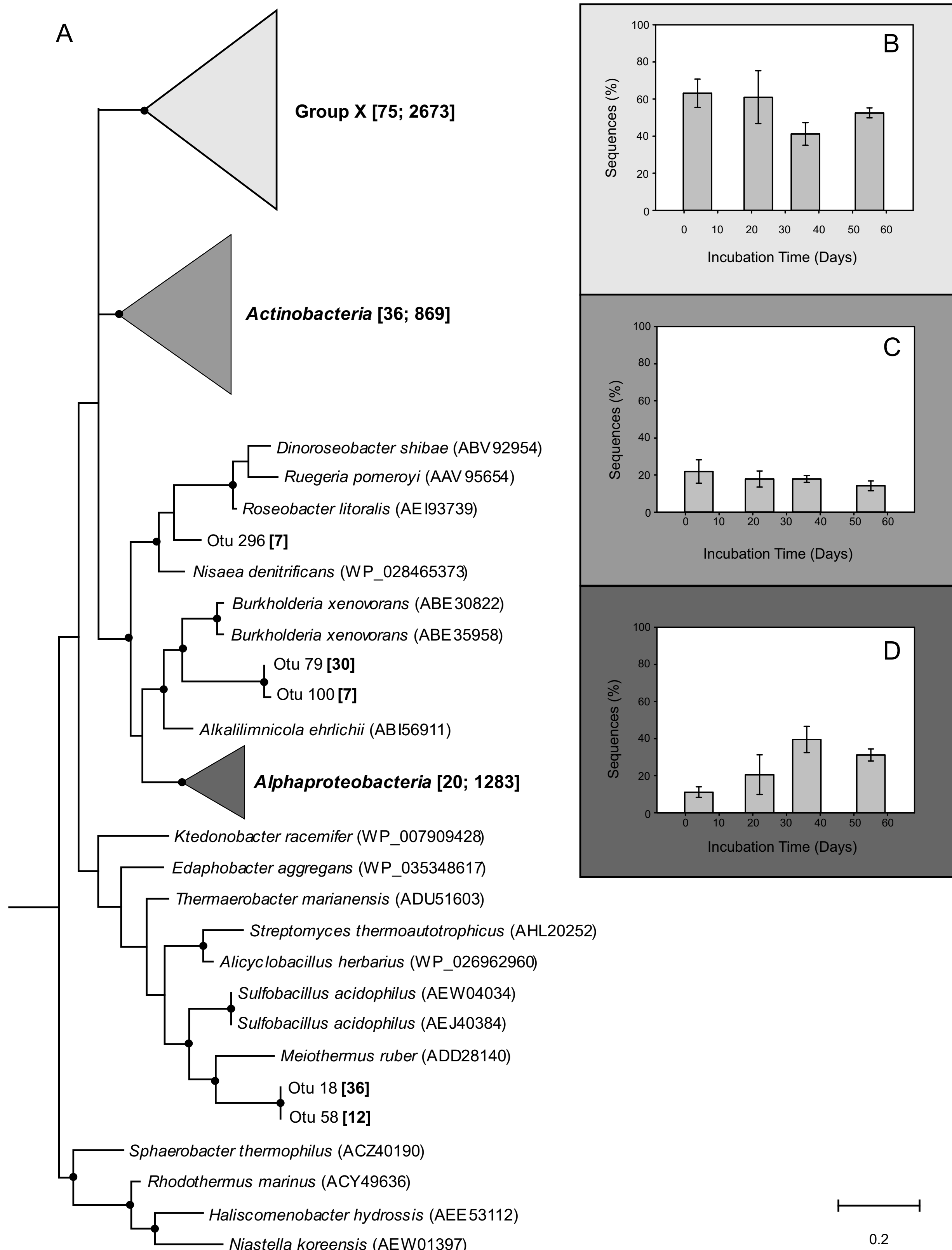

FIG 3 Maximum likelihood phylogenetic analysis of *coxL*-inferred amino acid sequences of 136 OTU and reference sequences from NCBI (147 residues). (A) The three main subclasses of sequences belonging to *coxL* are represented in the three clusters. The number of sequences of each OTU or a cluster is indicated in square brackets as "[OTU number; number of sequences]." The percentages of replicated trees in which the associated CoxL sequences clustered together in the bootstrap test (1,000 replicates) are shown for nodes supported by ≥50% of the replicates. (B to D) Time series of *coxL* sequence relative abundance in (B) *coxL* group x, (C) *coxL Actinobacteria*, and (D) *coxL Alphaproteobacteria* clusters.

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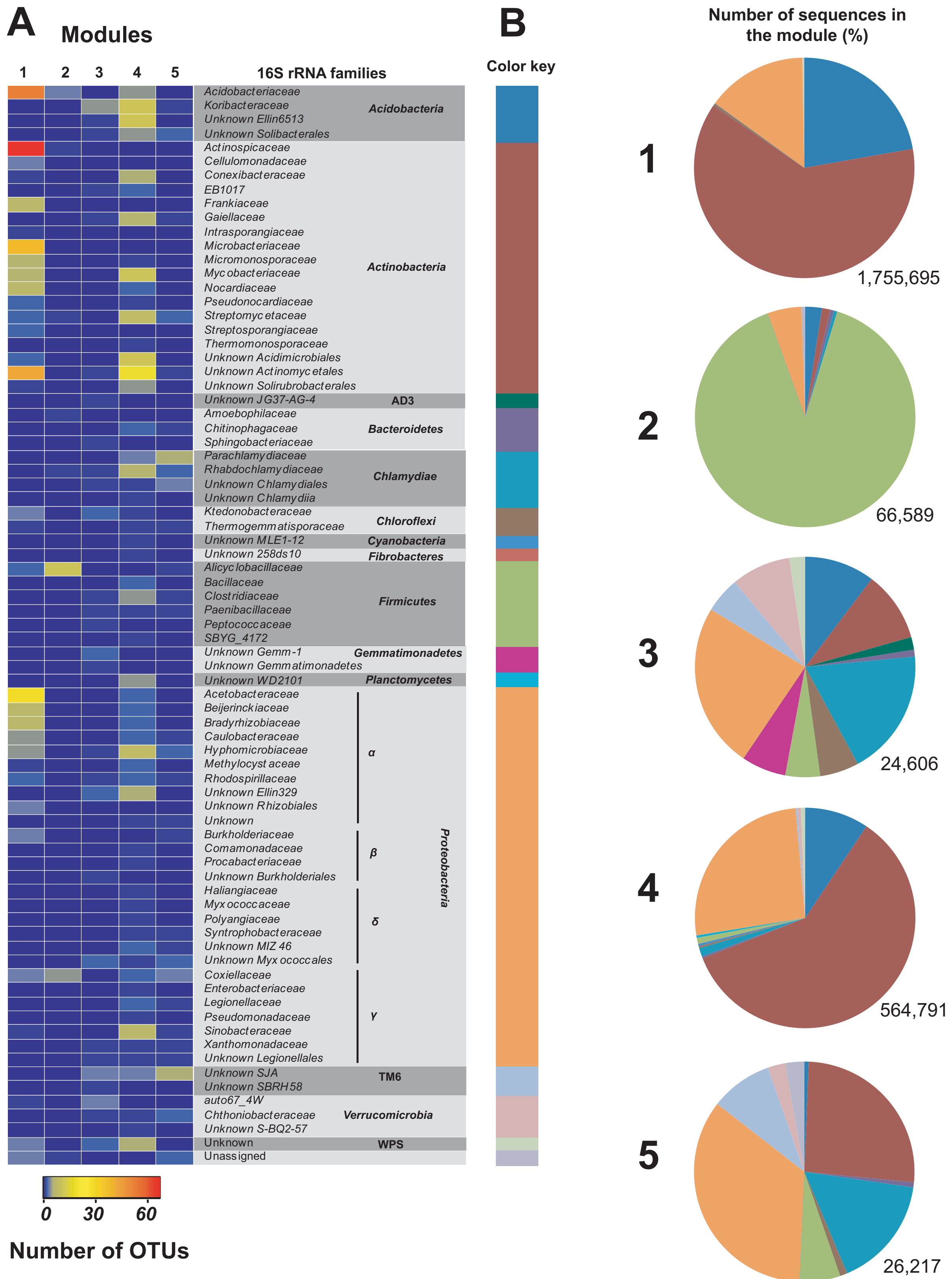

FIG 4 Taxonomic composition of the five modules defined by the covariance of the 16S rRNA OTU time series in the soil. (A) The heat map shows the number of OTU representing each family detected in the modules. (B) The OTU assigned to the same bacterial phyla were clustered together, and the number of sequences comprising each group was computed to report their relative abundance in the five modules. The color key used to identify each bacterial phylum in the pie charts is defined along the side of the heat map. The total number of sequences comprised in the modules is shown at the bottom of each pie chart.

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TABLE 2 Composition of module 1 comprising 16S rRNA OTU displaying colonization profiles related to maturation of CO oxidation activity in soil[a]

16S rRNA family	Abundance in module 1 (%)	Correlation[b]	Presence of *coxL*	Example accession no.[c]
Acidobacteriaceae	22.23	0.21*	+	WP_035348617
Unknown *Acidimicrobiales*	0.02	0.37	+	WP_035348617
Actinospicaceae	35.44	0.71**	−	NA[d]
Cellulomonadaceae	0.08	0.88***	−	NA
Conexibacteraceae	0.03	0.83***	+	ADB51446
Frankiaceae	0.42	0.29	−	NA
Intrasporangiaceae	0.01	0.09	−	NA
Microbacteriaceae	21.12	0.82***	−	NA
Micromonosporaceae	0.13	0.68*	+	WP_033364885
Mycobacteriaceae	0.97	−0.22	+	AFP37210
Nocardiaceae	0.31	0.22	+	WP_031936578
Pseudonocardiacea	0.09	0.78**	+	WP_028847129
Streptomycetaceae	0.19	0.05	+	WP_033825254
Streptosporangiaceae	0.46	0.59*	+	WP_020541099
Unknown *Actinomycetales*	2.70	0.61	+	WP_033364885
Unknown *Solirubrobacterales*	0.33	−0.19	+	ADB51446
Ktedonobacteraceae	0.29	−0.33	+	WP_007909428
Thermogemmatisporaceae	0.10	0.54	+	WP_052889641
Alicyclobacillaceae	0.03	0.28	+	WP_026962960
Acetobacteraceae	2.76	0.76**	−	NA
Beijerinckiaceae	2.39	0.45	+	WP_020174914
Bradyrhizobiaceae	7.43	0.79**	+	WP_014491996
Caulobacteraceae	1.27	0.36	−	NA
Hyphomicrobiaceae	0.13	0.85***	−	NA
Methylocystaceae	0.03	−0.63*	−	NA
Rhodospirillaceae	0.06	0.42	+	WP_028465373
Unknown *Rhizobiales*	0.07	0.93***	+	WP_014491996
Burkholderiaceae	0.50	0.60*	+	ABE35958
Coxiellaceae	0.05	0.22	−	NA
Sinobacteraceae	0.04	0.59*	−	NA
auto67_4W	0.03	0.31	NA	NA
Unknown WPS-2	0.20	0.84***	NA	NA
Unassigned	0.06	0.80**	NA	NA

[a] The relative abundance of sequences encompassing each family and the presence or the absence of the *coxL* gene in the reference genome of each taxonomic group are presented. Spearman correlations between the absolute abundance of OTU grouped at the family level and CO oxidation rate are presented.
[b] Correlation significance levels are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.
[c] Found by sequence identity with blastp (49) using the *coxL* sequence in *Burkholderia xenovorans* LB400 (accession no. ABE35958).
[d] NA, not applicable.

estimated by multiplying the relative abundance of the *coxL* OTU obtained from pyrosequencing profiles by the absolute abundance of *coxL* group x genes determined by qPCR. Considering the specific richness and the abundance of OTU encompassing the *coxL* group x genotype, module 1 was searched for potential 16S rRNA gene OTU candidates to infer a taxonomic affiliation with unknown presumptive carboxydovore bacteria. 16S rRNA OTU belonging to *Actinospicaceae* and *Microbacteriaceae* were the best *coxL* group x candidates, as predicted using the correlation network analysis. These two bacterial families were the sole candidates for which the absolute abundance and species richness of 16S rRNA OTU were congruent with those of *coxL* group x OTU in module 1 (see Table S1).

DISCUSSION

Enrichment of microorganisms utilizing atmospheric trace gas is challenging because the energy potential of these substrates usually supports mixotrophic survival metabolism, where reduced inorganic and organic compounds are simultaneously used as energy sources for maintenance or survival. Furthermore, increase of energy potential through increased gas concentration is not recommended because it will result in the enrichment of microbes that are incapable of scavenging atmospheric trace gas due to their low substrate affinity. Most high-affinity CO-oxidizing bacteria were identified through screening of isolates obtained from environmental samples or public collections after detection on *coxL* in sequenced genome. One notable exception was the isolation of the first carboxydovore bacterium, *Aminobacter* sp. strain cox1, after long-term exposure of soil to 40 to 400 ppmv CO (39). Recent investigations into the diversity of soil carboxydovore bacteria led to the identification of an atypical *coxL* group x genotype for which the distribution was significantly related to the CO uptake rate in soil (21). Before investment of cultivation efforts to isolate these unknown carboxydovore bacteria, we sought to challenge the hypothetical taxonomic affiliation of atypical *coxL* genotype to *Deltaproteobacteria* through the monitoring of microbial succession and maturation of CO uptake activity in soil microcosms.

Successions of 16S rRNA and *coxL* genes analyzed by qPCR

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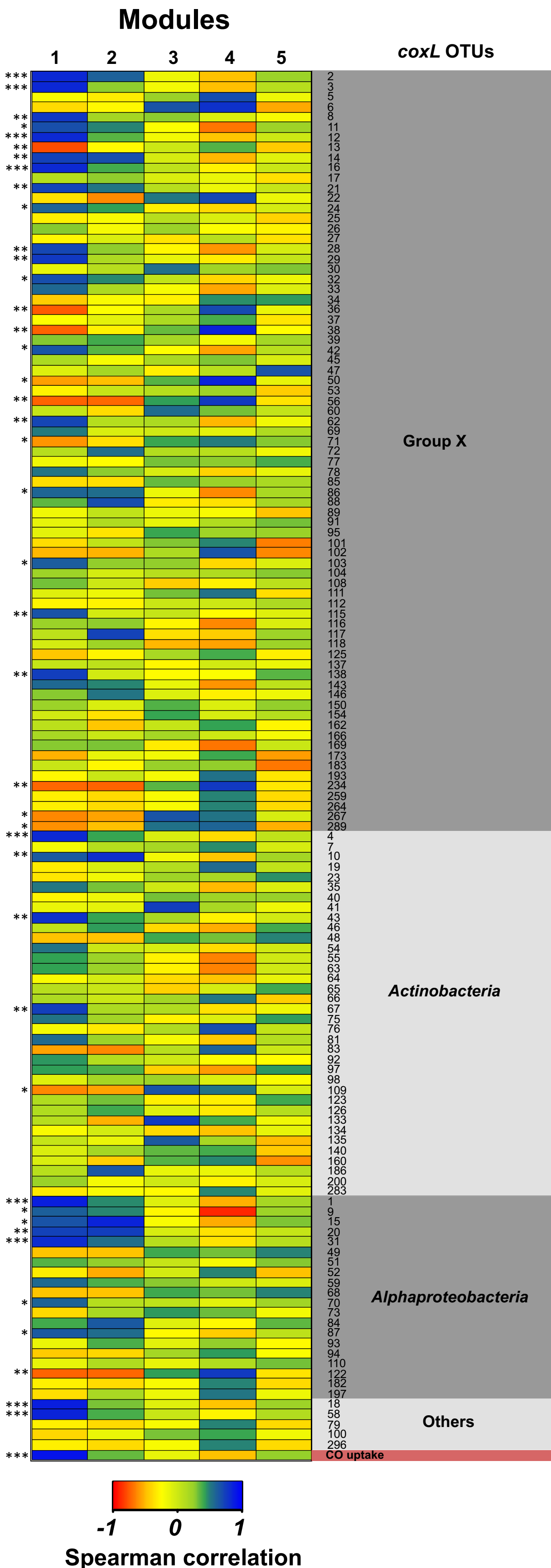

(Fig. 2) unveiled that *coxL* carboxydovore bacteria generally are opportunistic *r*-strategist bacteria using labile nutrients in soil. They also increase their relative abundance early in the incubation period. Our data showed that their enrichment was transient, suggesting that their populations were not maintained upon soil nutrient depletion. Interestingly, the CO-oxidizers represented by *coxL* group x displayed the *K*-strategist colonization profile, with slow growth of their population sustained by the efficient use of limited resources in soil. At the end of the incubation, total *coxL* and *coxL* group x genotypes represented 2.2% and 0.7% of the total bacterial population, respectively. It is believed that atmospheric CO can support mixotrophic metabolism of these carboxydovore bacteria (9). To address that question, we combined our qPCR time series with thermodynamic models. The theoretical population size of high-affinity CO-oxidizing bacteria in soil for which CO oxidation fully supplies maintenance energy requirements was estimated based on the theoretical maintenance energy consumption rate and the free energy of atmospheric CO oxidation, using the following model formulated by Conrad (40): $N = [1.4 \times 10^{14}\ (-\Delta G)\ d]/mE$, where N is the theoretical population size of CO-oxidizing bacteria in soil (expressed as the number of cells per gram of soil dry weight), 1.4×10^{14} is a constant expressing the density of bacterial cells containing 1 mol carbon (number of cells per mole of C biomass), ΔG is the Gibbs free energy of atmospheric CO oxidation (−235 kJ per mol of CO), d is the measured CO oxidation rate (moles of CO per gram of soil dry weight), and mE (4.5 kJ per mol of C biomass) is the energy maintenance requirement of the population (41). This model estimated that measured CO uptake activity would provide sufficient energy to support the survival of a maximal population 2.5×10^7 carboxydovore bacteria g dry weight $^{-1}$. According to our qPCR results, the abundance of total *coxL* and *coxL* group x sequences reached $(5.5 \pm 0.97) \times 10^9$ and $(1.8 \pm 0.20) \times 10^9$ copies g dry weight in soil at the end of the incubation, respectively. Taken together, these observations imply that oligotrophic cultivation media comprising both organic carbon and CO would be necessary to isolate unknown carboxydovore bacteria harboring the atypical *coxL* group x genotype that are expected mixotrophic *K*-strategists.

Although microbial succession was shaped by stochasticity in the colonial growth dynamic of individual cells (42) and alteration of the chemical structure and bioavailability of soil organic carbon due to autoclaving (43), the taxonomic affiliation of *coxL* genotypes inferred from phylogenetic analysis resulted in similar distributions compared with the observations made using native deciduous soil (21). *Actinobacteria*, *Alphaproteobacteria*, and *coxL* group x were the dominating *coxL* genotypes, representing 31% ± 3%, 14% ± 3%, and 53% ± 3% of the total sequences, respectively. Very few studies have reported the taxonomic composition of carboxydovore bacteria in soil. A soil survey along a succession of volcanic deposits unveiled an enrichment of *coxL* sequences closely related to *Burkholderia* isolates known to consume atmo-

FIG 5 Spearman correlations between the *coxL* OTU with the eigengenes of the five modules comprising 16S rRNA OTU displaying a similar soil colonization profile. Only the eigengene of module 1 was correlated with the CO uptake rate measured in soil microcosms. Significant correlations between the time series of individual *coxL* OTU and the eigengene of module 1 are marked with asterisks along the side of the heat map (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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spheric CO (10) as a function of vegetation regeneration and CO uptake activity (17, 44). In this study, two *coxL* OTU (OTU 79 and 100) related to *Burkholderia xenovorans* LB400 *coxL* sequence were detected, but their temporal distribution profile was not related to the maturation of CO uptake activity in soil. It has been reported that some species of mycobacteria from the *Mycobacteriaceae* family in the *Actinobacteria* phylum are capable of consuming atmospheric CO (45, 46). In this study, 14 *coxL* OTU closely related to *Mycobacterium* sequences were detected, but none displayed significant correlation with the CO oxidation rate in soil. The high abundance of *coxL* sequences closely related to *Bradyrhizobium* spp. and the correlation of their distribution profile with CO uptake activity suggest implication of these *Alphaproteobacteria* in the activity measured in the microcosms. Although they were not dominant in previous soil surveys, their capacity to consume atmospheric CO has already been reported using environmental isolates (10). Sequences encompassing *coxL* group x were abundant, with *H. ochraceum* as the only representative. CO uptake activity has been confirmed in this bacterium (21), but no *coxL* sequences have been noticed in sequenced genomes of the other aerobic *Deltaproteobacteria* encompassing the orders *Myxococcales* and *Bdellovibrionales*. The combination of qPCR and high-throughput sequencing of 16S rRNA and *coxL* genes led to the conclusion that it is very unlikely that *Deltaproteobacteria* harbor the *coxL* group x genotype. If unknown *Deltaproteobacteria* harbored the atypical *coxL* genotype, then a strong primer bias has impaired their detection or considerably underestimated their abundance.

Network correlation analysis led to the identification of taxonomic groups of bacteria for which the distribution was significantly correlated with the distribution of OTU encompassing *coxL* group x. Although the analysis does not imply any causal effect, it supported the suggestion that unknown carboxydovore bacteria are mixotrophic *K*-strategists, which is critical for the establishment of the best isolation strategy. Among the taxonomic groups detected in module 1 for which the eigengene was correlated to CO uptake rate, bacteria encompassing the *Actinospicaceae* and *Microbacteriaceae* families were the most probable taxonomic assignment of unknown carboxydovore bacteria harboring the *coxL* group x genotype. This taxonomic inference will need validation through isolation efforts since no representative of these two families was reported as CO-oxidizing bacteria. Interestingly, microorganisms that belong to these taxonomic groups are generally *K*-strategists, with some of them having the ability to use recalcitrant carbon such as cellulose (47). Furthermore, soil amendment experiments unveiled that cellulose stimulates CO uptake activity in soil, while glucose exerts the inverse effect (I. Lalonde, unpublished data). Bioprospection efforts are currently being used by the authors to identify and characterize unknown carboxydovore bacteria harboring the *coxL* group x genotype. Alternatively, single-cell sequencing (48) could be envisaged if cultivation efforts are unsuccessful.

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