Large Fractions of CO_2-Fixing Microorganisms in Pristine Limestone Aquifers Appear To Be Involved in the Oxidation of Reduced Sulfur and Nitrogen Compounds

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The traditional view of the dependency of subsurface environments on surface-derived allochthonous carbon inputs is challenged by increasing evidence for the role of lithoautotrophy in aquifer carbon flow. We linked information on autotrophy (Calvin-Benson-Bassham cycle) with that from total microbial community analysis in groundwater at two superimposed—upper and lower—limestone groundwater reservoirs (aquifers). Quantitative PCR revealed that up to 17% of the microbial population had the genetic potential to fix CO_2 via the Calvin cycle, with abundances of *cbbM* and *cbbL* genes, encoding RubisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) forms I and II, ranging from 1.14×10^3 to 6×10^6 genes liter^{-1} over a 2-year period. The structure of the active microbial communities based on 16S rRNA transcripts differed between the two aquifers, with a larger fraction of heterotrophic, facultative anaerobic, soil-related groups in the oxygen-deficient upper aquifer. Most identified CO_2-assimilating phylogenetic groups appeared to be involved in the oxidation of sulfur or nitrogen compounds and harbored both RubisCO forms I and II, allowing efficient CO_2 fixation in environments with strong oxygen and CO_2 fluctuations. The genera *Sulfuricella* and *Nitrosomonas* were represented by read fractions of up to 78 and 33%, respectively, within the *cbbM* and *cbbL* transcript pool and accounted for 5.6 and 3.8% of 16S rRNA sequence reads, respectively, in the lower aquifer. Our results indicate that a large fraction of bacteria in pristine limestone aquifers has the genetic potential for autotrophic CO_2 fixation, with energy most likely provided by the oxidation of reduced sulfur and nitrogen compounds.

Due to the lack of light-driven primary production, groundwater ecosystems were originally believed to be controlled by surface-derived allochthonous organic matter input (1–3) and to be dominated by heterotrophic prokaryotes adapted to nutrient limitation. However, there is increasing evidence of the important role of lithoautotrophy for carbon flow in aquifers (4–7). A large proportion of drinking water originates from groundwater resources (8), with karstic aquifers providing ~25% of the drinking water sources on a global scale (9). Despite the crucial role of microbial activity in shaping groundwater geochemistry (10–12), the links between microbial diversity and function in groundwater ecosystems, especially with regard to chemolithoautotrophy, are still poorly understood (7). Recent studies suggest that microbial CO_2 assimilation in aquifers could be fueled by energy conserved by nitrification, oxidation of ferrous iron and reduced sulfur compounds (6, 7), or oxidation of H_2 or methane (13, 14). Oxidation of electron donors present as solid minerals such as pyrite can even yield highly reactive dissolved ions that might affect other minerals and dissolved ions in the aquifer, leading to changes to the makeup of rocks and groundwater.

Today, there are six known autotrophic CO_2 fixation pathways (reviewed in reference 15): (i) the Calvin-Benson-Bassham cycle, (ii) the reductive tricarboxylic acid cycle, (iii) the reductive acetyl coenzyme A (acetyl-CoA) (Wood-Ljungdahl) pathway, (iv) the 3-hydroxypropionate cycle, (v) the 3-hydroxypropionate/4-hydroxybutyrate pathway, and (vi) the dicarboxylate/4-hydroxybutyrate cycle. The Calvin-Benson-Bassham cycle is quantitatively the most important mechanism of these pathways (15), and its key enzyme is ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), catalyzing the carboxylation of ribulose-1,5-bisphos-

phate to form two molecules of 3-phosphoglycerate. Four types of RubisCO are known to date. Bacteria utilize forms I and II (16, 17), which differ mainly with regard to their CO_2 affinity and CO_2/O_2 substrate specificity (18). RubisCO form II is thought to be the evolutionarily older form of RubisCO, with a lower CO_2/O_2 specificity and, thus, a lower tolerance toward oxygen (19). The *cbbL* and *cbbM* genes, encoding the large subunit of RubisCO forms I and II, respectively, have been widely used to analyze the diversity of CO_2-fixing chemolithoautotrophic bacteria in the environment. Such approaches revealed a wide distribution and diversity of RubisCO-encoding genes in soils (20–23) and groundwater (6, 7, 14, 24, 25). However, especially in aquifers, only a few studies investigated *cbbM* or *cbbL* genes at the transcript level or by quantitative approaches (7, 26).

Recent studies addressing the microbial diversity in aquifers or

drinking water wells by 16S rRNA gene-targeted pyrosequencing allowed comprehensive insight into the composition of microbial communities (14, 27–29). However, those studies were DNA based and did not allow characterization of active aquifer microbial community members, providing only limited insight into the physiologic strategies that are dominant under the given environmental conditions. Microbial activities strongly depend on aquifer physicochemical properties but in turn may also contribute substantially to shaping these properties, i.e., by oxidizing inorganic compounds to dissolved ions as part of a chemolithoautotrophic life-style.

During regular groundwater sampling campaigns in the Hainich region in Thuringia, central Germany, we sampled two superimposed pristine limestone aquifers: an upper, oxygen-deficient aquifer and a lower, oxygen-rich aquifer at a maximum depth of 88 m. We aimed to assess the metabolically active total and RubisCO-encoding microbial communities by targeting 16S rRNA, *cbbM*, and *cbbL* transcripts, and we monitored the temporal dynamics of these three genes in the two aquifers over 2 years. A marker gene-targeted approach was preferred over a more comprehensive metatranscriptomics approach as the latter requires large amounts of RNA starting material, which are inherently difficult to obtain from pristine groundwater, and detection of less abundant microbial community members might fail. We hypothesized (i) that the metabolically active microbial communities differ strongly between the two aquifers, driven mainly by the differences in oxygen availability; (ii) that the energy-providing metabolisms coupled to RubisCO-based autotrophy, as suggested by the phylogenetic affiliation of RubisCO-encoding microorganisms, also differ between the two aquifers; and (iii) that, assuming a reduced input of surface-derived organic material with increasing depth, putative autotrophs constitute a larger fraction of the microbial population in the lower than in the upper aquifer.

MATERIALS AND METHODS

Study site and sampling of groundwater. Groundwater samples were obtained from two superimposed limestone aquifers located in the Hainich region in northwest Thuringia. With a total size of 16,000 ha, the Hainich region is the largest connected deciduous forest in Germany, surrounded by agricultural areas of various management intensities. Groundwater was obtained from one well accessing an upper aquifer developed in the cycloides bank stratum (site H4-3 [12-m depth]) and two wells accessing a lower aquifer developed in a trochite limestone stratum (site H4-1 [48-m depth] and site H5-1 [88-m depth]). Sites were located on meadows (sites H4-1 and H4-3) and farmland (site H5-1), following a downhill slope with a distance of ~1,560 m between site H4 and site H5.

Prior to sampling, groundwater was pumped with a groundwater sampling pump (MP1; Grundfoss, Erkrath, Germany) until up to three well volumes were discharged and physicochemical parameters were stabilized. Samples for chemical analysis were filtered through 0.2-μm-pore-size filters and stored at 4°C until analysis. For molecular analyses, samples were filtered through 0.2-μm Supor filters (Pall Corporation), with 5 to 6 liters of groundwater passing through one filter. By integrating over a large volume of groundwater, we expected to capture a representative fraction of the microbial community. The filtration procedure lasted between 40 min and 90 min per filter, and water samples were kept cold during filtration. We cannot completely rule out that temporal exposure of the samples to oxygen during filtration may have caused shifts within the transcript pools. After filtration, filters were transferred into sterile reaction tubes and frozen on dry ice within <1 min. Filters were stored at −80°C until nucleic acid extraction.

Physicochemical analyses. Conductivity, pH, water temperature, oxygen concentration and saturation, and redox potential were measured electrometrically on-site with a flowthrough system using appropriate sensors (WTW, Weilheim, Germany). Dissolved CO$_2$ and bicarbonate concentrations were determined by titration directly after sampling according to Deutsche Einheitsverfahren (DEV) methods (30). Nitrate, nitrite, and ammonium levels were determined according to methods described previously by Velghe and Claeys (31) and Grasshoff et al. (32). Concentrations of Fe(II) were measured by the phenanthroline method (33). Concentrations of sulfate were determined by ion chromatography (IC 20 system [Dionex, Sunnyvale, CA] equipped with an IonPac AS11-HC column and an IonPac AG11-HC precolumn), and total organic carbon (TOC) concentrations were determined by using a TOC analyzer (AnalytikJena, Jena, Germany).

Nucleic acid extraction, amplification, clone library construction, and pyrotag sequencing. Genomic DNA and total RNA were isolated according to methods described previously by Church et al. (34), in combination with the DNA blood and tissue kit (Qiagen) and the RNeasy minikit (Qiagen). For each site, nucleic acids were extracted from one filter per time point. DNase treatment (Turbo DNase-free kit; Ambion, USA) and reverse transcription (RT) (Array Script reverse transcriptase; Ambion) was performed as previously described (35). Along with each sample, we prepared one control reaction mixture using the same RNA extract but without reverse transcriptase, which was subsequently PCR amplified to check for contamination of RNA extracts with genomic DNA.

Diversity analysis of both 16S rRNA and RubisCO-encoding genes was based on RNA only from groundwater samples obtained from the three wells at sites H4-3, H4-1, and H5-1 in November 2010 and April 2011. Analysis of transcripts of RubisCO-encoding *cbbL* genes was performed only for the samples obtained in April 2011. PCR amplification of *Bacteria* 16S rRNA fragments was performed by using primers 27F and 518R (36, 37), under the cycling conditions described in the supplemental material. Genes encoding RubisCO large subunit type IA (*cbbL*) and type II (*cbbM*) were amplified by using Hotstar *Taq* Mastermix (Qiagen) and primer pairs F-cbbL/R-cbbL and F-cbbM/R-cbbM, respectively, under cycling conditions reported previously (6). PCR products were checked by agarose gel electrophoresis and purified by using the NucleoSpin extract II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. For 16S rRNA and *cbbM* amplicons, barcode and linker sequences were added in a second PCR performed by GATC-biotech (Constance, Germany). Amplicons were sequenced with 454 pyrosequencing on a Roche GS FLX genome sequencer system by GATC-biotech. For analysis of *cbbL* transcript sequence diversity, we did not use next-generation sequencing but constructed clone libraries from RNA-based *cbbL* amplicons using the pGEM-T Easy vector and *Escherichia coli* JM109 chemically competent cells (Promega). The cloning approach was considered sufficient enough to assess the quantitatively dominant phyla potentially involved in CO$_2$ fixation and expressing *cbbL* genes. In addition, one clone library from DNA-based *cbbM* amplicons was constructed to generate standards for quantitative PCR (qPCR). Plasmid vector inserts of transformed *E. coli* clones were commercially sequenced (Macrogen Inc., South Korea).

Sequence analysis of 16S rRNA, *cbbM*, and *cbbL* amplicons. Pyrosequencing was performed with RNA-based 16S rRNA amplicons obtained from the three different wells sampled in November 2010 and April 2011. A total of 128,145 sequence reads were obtained for the 6 samples in total and were analyzed by using Mothur (38), along with SILVA bacterial reference alignment according to Schloss SOP (http://www.mothur.org/wiki/Schloss_SOP; see also the supplemental material) (39). For phylogenetic analysis of *cbbL* and *cbbM* transcripts, reference alignments were generated in ARB (40) (see the supplemental material). Nucleic acid sequences were transformed to deduced amino acid sequences for further phylogenetic analysis. Phylogenetic analysis of deduced CbbM and CbbL amino acid sequences of cultured representatives in ARB revealed that

sequence similarities between closely related species inferred from their relative positions in the calculated phylogenetic trees ranged from ~94 to 96%. Consequently, a 0.05 distance cutoff at the protein level was used for operational taxonomic unit (OTU) assignment of *cbbM* and *cbbL* sequences to allow for a good approximation of species-level distinctions. Analysis of RNA-based *cbbM* amplicons derived from pyrosequencing was performed by using Mothur, BioEdit (http://www.mbio.ncsu.edu /BioEdit/bioedit.html), and ARB (see the supplemental material). Assignment to OTUs and analysis of shared OTUs between samples were also conducted with Mothur. Coverages of the clone libraries were calculated according to methods described previously by Singleton et al. (41). Closest relatives were determined based on a BLAST search of representative OTU sequences, phylogenetic affiliations inferred from protein-based neighbor-joining trees, and distances calculated in ARB based on the clustering of sequences together with cultured representatives.

Quantitative PCR. Gene abundances of bacterial and archaeal 16S rRNA genes and of *cbbL* and *cbbM* genes were determined by quantitative PCR using Maxima SYBR green qPCR Mastermix (Fermentas) on an Mx3000P cycler (Agilent). 16S rRNA genes were amplified by using primer pair Bac8Fmod/Bac338EUB (42, 43), according to cycling conditions and standards described previously by Herrmann et al. (35). Functional genes were quantified by using primers F-cbbL/R-cbbL and F-cbbM/R-cbbM (6), as described in the supplemental material. Details on the estimation of relative fractions of the microbial population harboring RubisCO-encoding genes are provided in the supplemental material.

Statistical analysis. Differences in chemical parameters or gene abundances between sites were analyzed by using the t test or Mann-Whitney U test. Correlations between gene abundances and chemical parameters over time were analyzed by using the Spearman rank correlation coefficient. All statistical calculations were done with SPSS, version 19.0.

Nucleotide sequence accession numbers. Sequences obtained in this study were deposited in GenBank under accession numbers KF872279 to KF872456 (*cbbL* clone libraries) and KF872457 to KF872493 (*cbbM* clone library for qPCR standards). Pyrosequencing data for 16S rRNA and *cbbM* transcripts have been submitted to the European Nucleotide Archive under project number PRJEB5805 and sample accession numbers ERS420411 to ERS420422.

RESULTS

Biogeochemistry of the two aquifers. Previous investigations showed that the two aquifers harbored comparable microbial population densities, as revealed by SYBR green II total cell counts (site H4-3, 1.1×10^7 cells liter^{-1} to 9.4×10^7 cells liter^{-1} groundwater; site H4-1, 2.7×10^6 cells liter^{-1} to 3.8×10^8 cells liter^{-1}; site H5-1, 1.2×10^7 cells liter^{-1} to 3.7×10^8 cells liter^{-1}) (44). Groundwater oxygen saturation and nitrate concentrations were significantly lower in the upper aquifer (well H4-3, 5% O_2 and 6 µmol liter^{-1} nitrate) than in the lower aquifer (well H4-1, 37% O_2 and 121 µmol liter^{-1} nitrate; well H5-1, 38% O_2 and 137 µmol liter^{-1} nitrate). Maximum concentrations of sulfate (up to 3.20 mmol liter^{-1}) were observed at site H5-1, whereas sulfate concentrations were much lower in both aquifers at site H4 (Table 1). Groundwater from both aquifers had high ion contents (measured as conductivity), high concentrations of bicarbonate and dissolved CO_2, and a neutral pH slightly higher than 7 (Table 1). The TOC concentration was generally low but typical for pristine groundwater environments, ranging from 75 to 125 µmol liter^{-1}. Dissolved concentrations of inorganic electron donors were rather low, with mean concentrations of NH_4^+ ranging from 4.6 to 9.0 µmol liter^{-1} (Table 1) and concentrations of Fe(II) usually being <5 µmol liter^{-1} (data not shown). When water was sampled for community analyses, physicochemical parameters were similar to the mean values over a 2-year period (Table 1).

TABLE 1 Physicochemical parameters of the groundwater at site H4 with two wells and further downstream at site H5[a]

Site and date	Mean conductivity (µS cm^{-1}) ± SD	Mean pH ± SD	Mean oxygen saturation (%) ± SD	Mean dissolved CO_2 concn (mmol liter^{-1}) ± SD	Mean bicarbonate concn (mmol liter^{-1}) ± SD	Mean TOC concn (µmol liter^{-1}) ± SD	Mean NO_3^- concn (µmol liter^{-1}) ± SD	Mean NH_4^+ concn (µmol liter^{-1}) ± SD	Mean SO_4^{2-} concn (mmol liter^{-1}) ± SD
H4-3									
24-mo study period	747 ± 27	7.2 ± 0.1	5.3 ± 9.9	0.46 ± 0.17	6.74 ± 0.63	94.9 ± 9.2	6.0 ± 6.8	7.8 ± 3.0	0.37 ± 0.1
Nov 2010	645	7.3	1.0	ND	ND	112.4	BD	10.8	0.39
Apr 2011	754	7.2	3.5	0.45	7.55	83.3	BD	5.5	0.41
H4-1									
24-mo study period	748 ± 50	7.3 ± 0.1	38.3 ± 15.0	0.39 ± 0.18	6.33 ± 0.79	74.1 ± 10.8	121.2 ± 51.7	9.0 ± 6.7	0.79 ± 0.32
Nov 2010	656	7.4	39.6	ND	ND	68.3	142.6	11.4	0.76
Apr 2011	776	7.3	62.8	0.39	5.81	81.6	160.0	BD	1.09
H5-1									
24-mo study period	1013 ± 72	7.1 ± 0.1	37.1 ± 9.1	0.44 ± 0.18	5.43 ± 0.56	79.1 ± 10.0	137.3 ± 22.2	4.6 ± 4.5	2.53 ± 0.34
Nov 2010	842	7.2	40.7	ND	ND	87.4	186.8	2.9	2.09
Apr 2011	1051	7.2	36.8	0.44	5.47	94.9	131.0	BD	2.64

[a] Data are from site H4 with two wells (wells H4-3 [12-m depth] and H4-1 [48-m depth]) and further downstream at site H5 (well H5-1 [88-m depth]). Data are means ± standard deviations of results obtained during a 24-month study period. For each site, values are also given for the months when samples were taken for diversity analysis. Well H4-3 was in the upper cycloides bank aquifer, whereas wells H4-1 and H5-1 were located in the lower trochite limestone aquifer. Detection limits for NO_3^- and NH_4^+ were 1 µmol liter^{-1} and 0.5 µmol liter^{-1}, respectively. ND, not determined; BD, below the limit of detection.
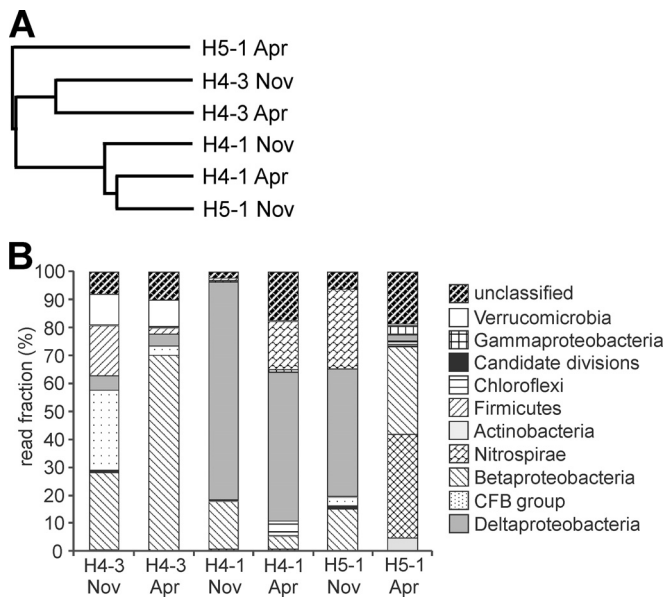
FIG 1 Results of 16S rRNA-targeted pyrosequencing. (A) Dendrogram based on Bray-Curtis similarity values; (B) phylogenetic affiliation at the phylum level. Only phyla represented by >1% of the sequence reads in at least one sample are shown. Candidate divisions include OD1, OP10, OP3, TG-1, TM7, WS3, BD1-5, and WCHB1-60. The CFB group includes members of the *Cytophagales*, *Flavobacteria*, and *Bacteroidetes*.

Diversity of active groundwater bacterial communities (16S rRNA). We analyzed sequences of bacterial 16S rRNA, *cbbM*, and *cbbL* gene transcripts to link RubisCO-based autotrophy to physiologies inferred from closely related described taxa within active groundwater microbial communities. 16S rRNA diversity was assessed by 454 pyrosequencing of RNA-based 16S rRNA amplicons for samples obtained in November 2010 and April 2011. For comparative analysis, we utilized the data set after normalization to the lowest number of reads found in sample H5-1 from April. More information is given in the supplemental material. Comparison of samples using Bray-Curtis indices revealed that samples from the upper aquifer (site H4-3) obtained at two different time points were most similar and that sample H5-1 from April, which had the

highest level of diversity, was the most different from the other samples (Fig. 1A and Table 2).

The majority of sequences (69% of total sequences) were related to members of the *Proteobacteria*, including *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, and *Epsilonproteobacteria* (Fig. 1B). Members of the *Deltaproteobacteria* represented the highest number of sequence reads in almost all the samples obtained from the lower aquifer (46% of sequences from site H5-1 in November, 53% from site H4-1 in April, and 78% from site H4-1 in November) (Fig. 1B). *Nitrospirae*-affiliated phylotypes represented 29% and 17% of sequences in samples from the lower aquifer in November 2010 (site H5-1) and in April 2011 (site H4-1), respectively. In contrast, members of the *Betaproteobacteria* and the CFB (*Cytophagales*, *Flavobacteria*, and *Bacteroidetes*) group dominated the active community in the upper aquifer, together representing 56% and 73% of all sequences in November 2010 and April 2011, respectively (Fig. 1B).

The 25 most abundant OTUs accounted for ~70% of all sequence reads. The highest number of overall sequence reads (21%) belonged to an OTU assigned to the *Deltaproteobacteria* with a sequence similarity to cultured microorganisms of <90%. This OTU accounted for a large fraction (37 to 44%) of sequence reads in the lower aquifer samples but was almost absent from the upper aquifer. It had 99% and 97% sequence similarities to environmental sequences from lake sediment (GenBank accession number AB661566 [45]) and groundwater (accession numbers KC606878 [46] and DQ407360 [47]), respectively. The second most abundant OTU (7% of total sequence reads) was related to *Nitrospira moscoviensis* (93.5% sequence identity). The two *Nitrospira*-related phylotypes found in our study were most closely related to environmental sequences obtained from beech forest soil (48) or from grassland soil (49). Abundant OTUs representing large fractions of sequences in samples from the upper aquifer (site H4-3) were related to the genera *Pedobacter* (up to 23.9%), *Limnohabitans* (up to 17%), *Albidiferax* (up to 16.1%), *Opitutus* (up to 10.1%), and *Polaromonas* (up to 8.6%); however, these groups represented <0.8% of total sequences in the lower aquifer (sites H4-1 and H5-1). Two OTUs closely related to *Sulfuricella denitrificans* (98% sequence identity) and *Nitrosomonas* sp. strain Is79A3 (98.4% sequence identity), groups known to fix CO$_2$ via the Calvin-Benson-Bassham cycle (17, 50), were also detected in

TABLE 2 Overview of pyrosequencing data sets for 16S rRNA and *cbbM* transcripts after normalization[a]

Sample and mo	Gene	No. of sequences	Coverage	Inverse Simpson's index (1/D)	No. of observed OTUs	No. of estimated OTUs (chao)	No. of rare OTUs (singletons)	Shannon index
H4-3 Nov	16S rRNA	7,317	0.92	9.16	797	3,062	727 (610)	3.36
H4-3 Apr	16S rRNA	7,317	0.92	13.79	784	2,612	695 (571)	3.74
H4-1 Nov	16S rRNA	7,317	0.95	4.27	539	1,755	479 (395)	2.65
H4-1 Apr	16S rRNA	7,317	0.94	6.04	644	1,940	555 (453)	3.19
H5-1 Nov	16S rRNA	7,317	0.96	3.85	401	1,268	355 (289)	2.28
H5-1 Apr	16S rRNA	7,317	0.85	60.25	1,537	5,301	1,341 (1,115)	5.46
H4-3 Nov	*cbbM*	4,830	0.99	10.21	97	250	64 (53)	2.81
H4-3 Apr	*cbbM*	4,830	0.99	1.61	63	141	47 (38)	0.97
H4-1 Nov	*cbbM*	4,830	0.99	7.78	95	195	58 (43)	2.59
H4-1 Apr	*cbbM*	4,830	0.99	1.97	71	102	45 (30)	1.41
H5-1 Nov	*cbbM*	4,830	0.99	5.95	93	159	59 (42)	2.32
H5-1 Apr	*cbbM*	4,830	0.99	4.50	60	192	41 (33)	1.97

[a] Sequence distance cutoffs for OTU assignment were 0.03 (nucleic acid level) for 16S rRNA genes and 0.05 (protein level) for *cbbM*.
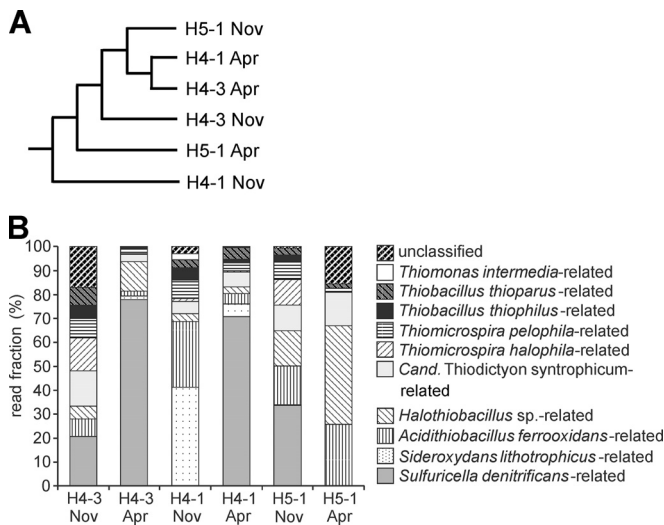
A

B

FIG 2 Results of *cbbM*-targeted pyrosequencing, based on deduced *cbbM* protein sequences. (A) Dendrogram based on Bray-Curtis similarity values; (B) phylogenetic affiliation and relative frequency of the different sequence types. Only phylotypes represented by >1% of the sequence reads in at least one sample are shown. Closest relatives were determined based on BLAST results and distances calculated in ARB. Similarities of deduced *cbbM* protein sequences obtained in this study to cultivated strains are as follows: 90.4 to 97.3% for *Sulfuricella denitrificans*, 91.1 to 96.2% for *Sideroxydans lithotrophicus* ES-1, 90.5 to 97.3% for *Acidithiobacillus ferrooxidans*, 90.4 to 94.7% for *Halothiobacillus* sp., 91 to 98.7% for "*Candidatus* Thiodictyon," 90.4 to 97.3% for *Thiomicrospira halophila*, 90.4 to 98.6% for *Thiomicrospira pelophila*, 90.5 to 94.6% for *Thiobacillus thiophilus*, 90.7 to 97.3% for *Thiobacillus thioparus*, and 90.4 to 97.3% for *Thiomonas intermedia*.

the 16S rRNA read pool from the lower aquifer, representing 0.8 to 5.6% (*Sulfuricella*) and 0.3 to 3.8% (*Nitrosomonas*) of total reads.

Diversity of the CO₂-fixing microbial community based on *cbbM* and *cbbL* transcripts. The composition of the microbial community transcribing *cbbM* genes was analyzed by 454 pyrosequencing for samples obtained in November 2010 and April 2011. Comparison of samples by using Bray-Curtis indices based on normalized data sets did not result in an obvious clustering pattern according to site or time (Fig. 2A). Only 7 of the 324 total OTUs were shared by communities of all samples; however, 4 of these OTUs accounted for ~60% of all sequence reads and were detected at all three wells at both sampling times. The most abundant OTUs were related to *Sulfuricella denitrificans* (97.3% similarity at the protein level; 9,745 reads), *Halothiobacillus* sp. (94.6% similarity; 3,014 reads), *Acidithiobacillus ferrooxidans* (97.3% similarity; 2,593 reads), *Sideroxydans lithotrophicus* ES-1 (92.4 to 93.6% identity; 2,200 reads), and "*Candidatus* Thiodictyon syntrophicum" (98.7% identity; 2,005 reads) (Fig. 2B; see also Fig. S4

in the supplemental material). However, the relative fractions of sequence reads of each phylotype differed considerably between sampling sites or time points. While *Sulfuricella*-related sequence reads constituted >70% of all reads for samples from sites H4-3 and H4-1 in April 2011, this phylotype was represented by only a few reads in samples obtained from site H4-1 in November 2010 and from site H5-1 in April 2011 (Fig. 2B). Sequences related to *Thiobacillus thioparus*, *Halothiobacillus neapolitanus*, and *Thiomicrospira pelophila* usually formed only a small fraction of the *cbbM* transcript pool. Overall, the composition of the active *cbbM*-transcribing community did not show a pattern that would suggest that *cbbM* OTU patterns were related to the aquifer or sampling time point.

The active CO₂-fixing microbial community harboring *cbbL* genes was analyzed by using a cloning approach targeting *cbbL* transcripts for one sampling time per well (April 2011). Using a 0.05 distance cutoff for deduced *cbbL* protein sequences, we detected 13 OTUs in total (see Fig. S5 in the supplemental material). Coverage ranged from 96.7 to 98.3% for 58 to 60 sequences per library (Table 3), indicating that the cloning approach sufficiently covered the major phylotypes. Similar to the *cbbM*-targeted approach, the 6 shared OTUs represented at least 70% of the sequences within each sample and 74% of total sequence reads. While the *cbbL* genotypes were dominated by sequences related to *Sulfuricella denitrificans* in the upper aquifer (site H4-3; ~43%), the *Sulfuricella*-related fraction represented only 10 to 20% of sequences in the lower aquifer (Fig. 3B). In turn, ~30% of genotypes in the lower aquifer were related to the ammonium-oxidizing bacterium *Nitrosomonas* sp. IsA73 (GenBank accession number YP_004694496), which was represented by only a few clones in the upper aquifer. This pattern was reflected by a higher level of similarity between samples obtained from the two wells of the lower aquifer (Fig. 3A). *Sideroxydans lithotrophicus* ES-1-related sequences formed a comparably large fraction at all three wells, while *Acidithiobacillus ferrivorans*-related sequences formed a larger fraction at site H5-1.

Temporal variation of the abundance of RubisCO-encoding and 16S rRNA genes. The abundance of potential CO₂ fixers using RubisCO and the total prokaryotic population was approximated from analysis of RubisCO-encoding and 16S rRNA genes in genomic DNA extracted from groundwater. Numbers of *cbbM* genes, encoding RubisCO form II, ranged from 5.04×10^3 to 5.93×10^6 genes per liter of groundwater over a 2-year period, while numbers of *cbbL* genes, encoding RubisCO form IA, were between 2 and 8 times lower (ranging from 1.14×10^3 to 1.75×10^6 genes liter^{-1}) (Fig. 4). Substantial temporal fluctuations of *cbbL* and *cbbM* gene abundances of 2 orders of magnitude at each site were observed but without significant differences in the abundances of these genes between the upper and lower aquifers or

TABLE 3 Overview of clone library-based analysis of *cbbL* transcripts[a]

Sample and mo	No. of sequences	Coverage (%)	Inverse Simpson's index (1/D)	No. of observed OTUs	No. of estimated OTUs (chao)	Shannon index
H4-3 Apr	60	96.7	7.53	9	10	2.00
H4-1 Apr	60	98.3	8.12	11	11	2.16
H5-1 Apr	58	98.3	5.01	8	8	1.73

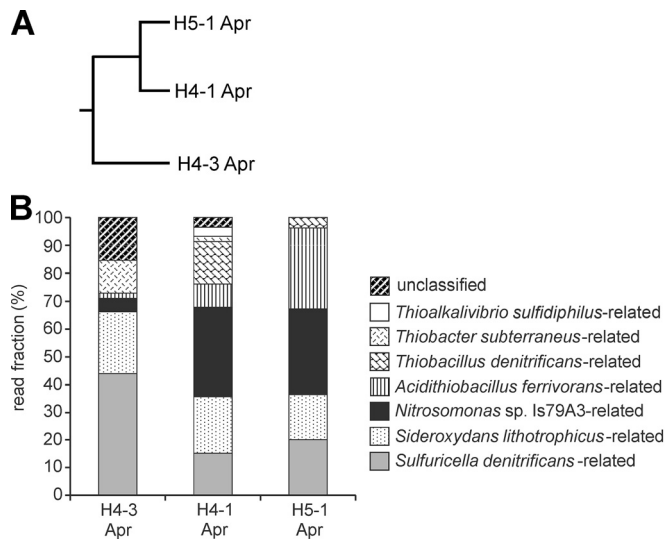[a] Sequence distance cutoffs for OTU assignment were 0.05 (protein level).

FIG 3 Results of the *cbbL* cloning approach, based on deduced *cbbL* protein sequences. (A) Dendrogram based on Bray-Curtis similarity values; (B) phylogenetic affiliation and relative frequency of the different sequence types. Closest relatives were determined based on BLAST results and distances calculated in ARB. The upper aquifer included site H4-3, and the lower aquifer included sites H4-1 and H5-1. Similarities of deduced *cbbL* protein sequences obtained in this study to cultivated strains are as follows: 95.6 to 100% for *Sulfuricella denitrificans*, 95.5 to 98.3% for *Sideroxydans lithotrophicus* ES-1, 96.7 to 99.4% for *Nitrosomonas* sp. Is79A3, 94.4 to 96.1% for *Acidithiobacillus ferrivorans*, 92.2 to 96.7% for *Thiobacillus denitrificans*, 92.2 to 95.5% for *Thiobacter subterraneus*, and 91.6% for *Thioalkalivibrio sulfidiphilus*.
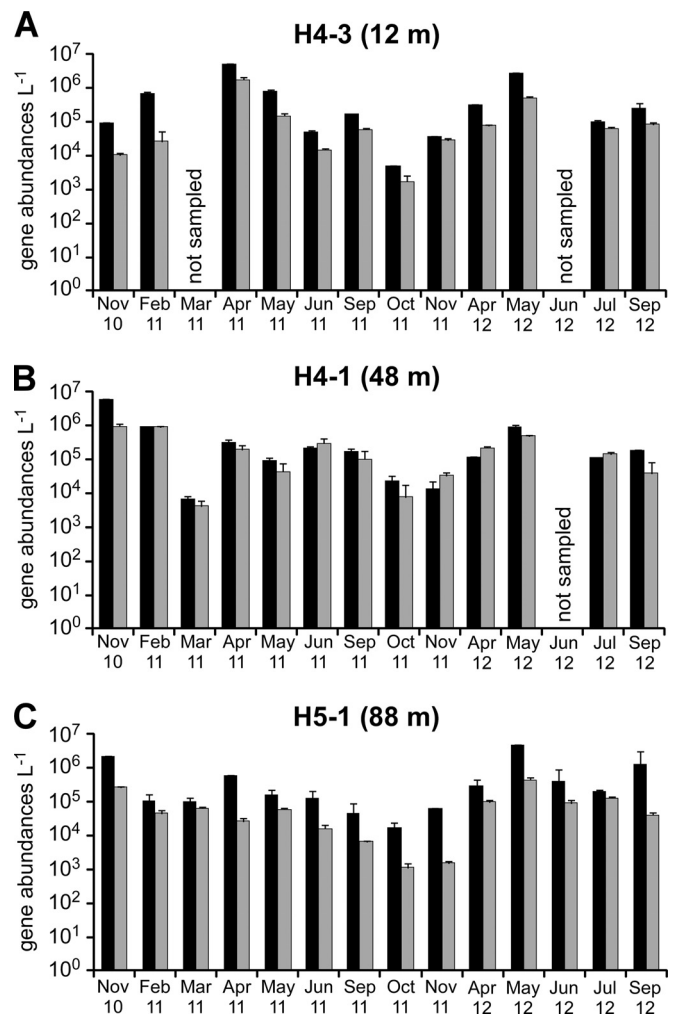

FIG 4 Abundance of *cbbM* (black) and *cbbL* (gray) genes per liter groundwater over a 2-year period. (A) Site H4-3 (upper aquifer; 12-m depth); (B) site H4-1 (lower aquifer; 48-m depth); (C) site H5-1 (lower aquifer; 88-m depth). Error bars represent the standard deviations of data from three replicate qPCRs.

between the two wells of the lower aquifer. Groundwater bacterial 16S rRNA gene numbers, providing an approximation of the total microbial population, ranged from 3.11×10^5 to 2.69×10^8 genes liter^{-1} at the three different sites, as reported previously (44). Archaeal 16S rRNA genes were ~2 orders of magnitude less abundant than bacterial 16S rRNA genes, and their numbers ranged from 1.3×10^3 to 4.6×10^5 genes liter^{-1} (44). Similar to the RubisCO-encoding genes, there were strong temporal fluctuations but no significant differences among the three sampling sites. Estimated cell numbers based on bacterial and archaeal 16S rRNA gene qPCR data were in the same range as the total cell counts performed on selected samples by Opitz et al. (44).

For each well, *cbbM* and *cbbL* gene numbers were positively correlated with each other (Spearman rank correlation coefficients of 0.84 for site H4-3, 0.89 for site H4-1, and 0.72 for site H5-1; $P < 0.01$) and with bacterial 16S rRNA gene numbers (Spearman rank correlation coefficients of 0.89 for *cbbM* and 0.85 for *cbbL*; $P < 0.001$) over time. Based on 16S rRNA, *cbbM*, and *cbbL* gene numbers, the estimated fraction of organisms of the total community with the genetic potential to assimilate CO$_2$ via the Calvin-Benson-Bassham cycle ranged from 0.5 to 14.4% in the upper aquifer (site H4-3) and from 2.1 to 17.3% in the lower aquifer (sites H4-1 and H5-1).

Temporally, the abundances of *cbbM* and *cbbL* genes showed maxima in spring and early summer, were low in autumn (Fig. 4), and thus followed the same patterns as those previously observed for bacterial 16S rRNA genes for the same samples (44). *cbbM*-to-16S rRNA gene ratios exhibited variations of usually <1 order of magnitude in both aquifers over time (Fig. 5), indicating that CO$_2$-fixing microorganisms harboring *cbbM* genes made up a

rather stable fraction of the total microbial community. However, *cbbL*-to-16S rRNA gene ratios were subject to substantial temporal variations in the lower aquifer at site H5-1, with minima from June to November 2011 (Fig. 5), suggesting stronger changes in the CO$_2$-fixing community over time than in upstream well H4-1 or the upper aquifer (site H4-3). *cbbM* and *cbbL* gene abundances did not show significant correlations with oxygen saturation or CO$_2$ concentrations over time, except for site H5-1 of the lower aquifer, where *cbbM* gene abundances were negatively correlated with oxygen saturation (Spearman rank correlation coefficient of -0.55; $P < 0.05$) and both *cbbM* and *cbbL* gene abundances were positively correlated with CO$_2$ concentrations (Spearman rank correlation coefficients of 0.60 and 0.70, respectively; $P < 0.05$).

Transcript-to-gene ratios of bacterial 16S rRNA, *cbbM*, and *cbbL* at selected time points were analyzed by qPCR (see Fig. S6 in the supplemental material). While transcript-to-gene ratios were usually between 1 and 10 for bacterial 16S rRNA, maximum estimates of transcript-to-gene ratios for *cbbM* and *cbbL* ranged from
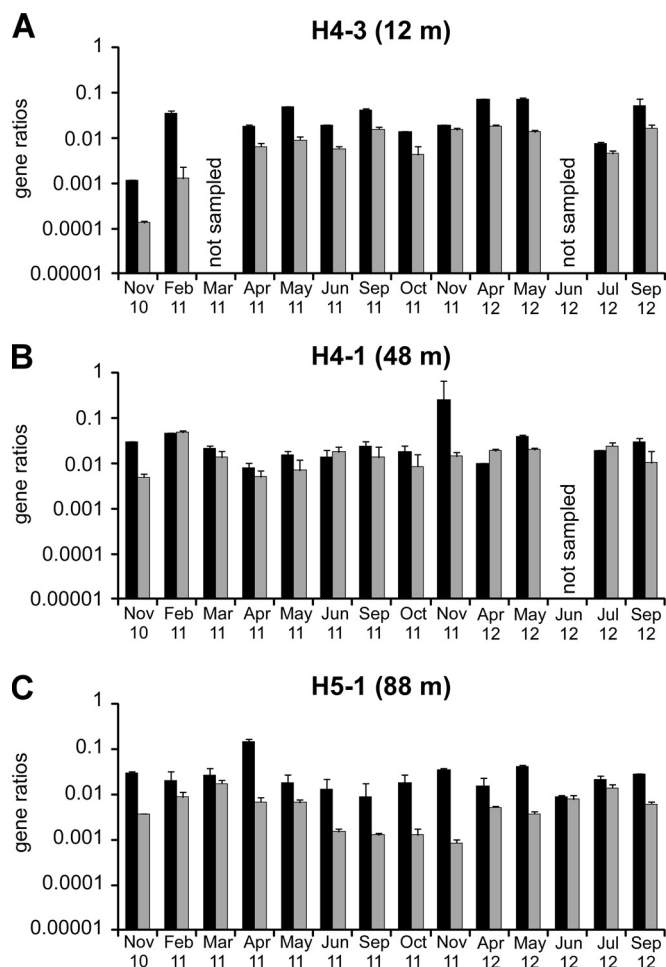
FIG 5 Relative numbers of *cbbM* (black) and *cbbL* (gray) genes compared to bacterial and archaeal 16S rRNA gene numbers over a 2-year period. (A) Site H4-3 (upper aquifer; 12-m depth); (B) site H4-1 (lower aquifer; 48-m depth); (C) site H5-1 (lower aquifer; 88-m depth). Error bars represent the standard deviations of data from three replicate qPCRs.

0.38 to 0.01, corresponding to $<1 \times 10^5$ *cbbM* or *cbbL* transcripts per liter groundwater.

DISCUSSION

Structure of active bacterial communities. Factors that affect microbial diversity and abundance in groundwater ecosystems were previously linked to the interplay between organic contaminants, nutrients, and hydrogeology (8, 51–53). In addition, seasonal hydrological dynamics, e.g., resulting from snowmelt, may have strong effects on groundwater microbial community composition (54). In the system of two superimposed limestone aquifers investigated in this study, both aquifers were characterized as pristine, with low concentrations of organic carbon and nutrients and the same general lithology but with strong differences in oxygen concentrations. In contrast to our expectations, we did not observe a decrease in the concentration of TOC with increasing depth. This might be because the input of TOC is similarly low for both aquifers. Alternatively, assuming a higher input of TOC into the upper than in the lower aquifer, higher TOC turnover rates in the upper aquifer might result in the observed equally low TOC concentra-

tions in both aquifers. The latter would be supported by the lower oxygen availability in the upper aquifer. Oxygen availability and distance to surface soils were likely key drivers of the observed differences between the compositions of the active microbial communities in the upper and the lower aquifers. In support of our hypothesis, we found little overlap in the active bacterial communities between the two aquifers. Members of the *Betaproteobacteria* and *Cytophagales-Flavobacteria-Bacteroidetes* group dominated the metabolically active communities in the upper aquifer. The dominance of *Betaproteobacteria* in groundwater agrees with data from previous reports (29, 55–57). Sequences related to facultative or obligate anaerobes, e.g., the genera *Albidiferax*, *Limnohabitans*, and *Opitutus*, together represented 26.2 to 30.5% of the sequences reads in the total active community in the upper aquifer, which reflects the oxygen-deficient conditions of this aquifer. Metabolically active communities in the upper aquifer also harbored a larger fraction of soil-related genera, e.g., *Pedobacter*, *Opitutus*, *Albidiferax*, and *Polaromonas* (58–61), belonging to the families *Sphingobacteriaceae*, *Opitutaceae*, and *Comamonadaceae*. In fact, a recent study of microbial communities in seepage water of agricultural soil revealed that representatives of these bacterial families originating from the rhizosphere and rhizoplane were subject to vertical migration (62). Consequently, large fractions of the bacterial community in the upper aquifer might actually originate from surface soils and could be preferentially transported vertically to the groundwater by event-driven transport upon heavy rainfalls or snowmelt (62, 63).

The metabolically active communities in the lower, oxygen-rich aquifer showed a very different structure and harbored large fractions of *Deltaproteobacteria* and *Nitrospirae*. The large proportions of *Nitrospirae* are similar to those reported for alpine karstic spring water (64) and granitic aquifers with low oxygen availability (65). *Deltaproteobacteria* are occasionally reported in groundwater environments (66) but in most cases represent sulfate reducers under conditions of low oxygen availability (46). Due to the low level of sequence similarity of the most abundant *Deltaproteobacteria*-related OTU found in our study, we cannot infer the potential metabolism of these organisms. The second most abundant OTU was related to *Nitrospira moscoviensis*, with 93.5% sequence identity, which agrees with data from previous reports of the presence of *Nitrospira*-related phylotypes in water of an alpine radioactive thermal spring (67), suggesting a role in nitrification. Moreover, the distribution pattern of this OTU, with a much higher relative abundance in the lower aquifer at higher nitrate concentrations than in the upper aquifer, would also support its role in nitrification.

Despite the strong differences in the compositions of the metabolically active microbial communities, total microbial abundances were not affected by the differences in physicochemistry between the two aquifers, as both aquifers harbored comparable microbial population densities. In fact, the microbial abundance estimated by 16S rRNA gene qPCR previously reported for the same samples (44) was comparable to those in limestone spring water (64) and two alpine deep granitic groundwater sites (65) but was 1 to 2 orders of magnitude lower than those in other granitic aquifers (57, 68).

Chemolithoautotrophy linked to sulfur and nitrogen cycling. Given the differences in oxygen availability and microbial community composition between the two aquifers, we expected to find that the active microbial communities transcribing *cbbM*

or *cbbL* genes would differ strongly between the two aquifers. In fact, there was a clear separation of the two aquifers at the level of *cbbL* but not *cbbM* transcripts. The lack of clustering at the level of *cbbM* transcripts could be linked to the fact that most of the dominant *cbbM* phylotypes belonged to microaerophilic or facultative anaerobic microorganisms able to thrive under the conditions of both aquifers. In addition, the composition of the CO$_2$-fixing microbial community transcribing *cbbM* genes varied strongly between the November 2010 and April 2011 samples, suggesting substantial changes over time. The reasons for these changes remain speculative and could be linked to the availability of anoxic microsites within the karstic aquifer system.

Most of the RubisCO-expressing phyla detected in our study harbored both RubisCO forms I and II, which agrees with previous findings from groundwater environments (25). The existence of both form I and form II RubisCO in one organism has been explained by the different kinetic properties of the enzymes, allowing efficient CO$_2$ fixation in environments with strong fluctuations in the levels of oxygen and CO$_2$ (6). RubisCO form I is capable of selectively fixing CO$_2$ regardless of the presence of oxygen and therefore is better adapted to lower CO$_2$ concentrations and/or oxic conditions (19, 69, 70). The simultaneous expression of both *cbbL* and *cbbM* by some organisms in groundwater with oxygen saturation of 5 to 63% observed here is in contrast to findings of Alfreider and coworkers (7), who did not detect *cbbM* transcripts in oxygen-amended groundwater, but agrees with data from previous reports of cultivation-based studies (71, 72).

Most of the *cbbL* and *cbbM* phylotypes detected in this study were closely related to the genera *Sulfuricella*, *Sideroxydans*, and *Acidithiobacillus*, pointing to a strong link between autotrophy and the oxidation of reduced sulfur compounds, as previously reported for groundwater environments (6, 7, 25, 26). Since representatives of the genera *Sideroxydans* and *Acidithiobacillus* are capable of oxidizing Fe(II), chemolithoautotrophy in the two aquifers could also be coupled to iron oxidation. However, given the low concentrations of Fe(II) in the groundwater of both aquifers along with the prevailing oxic conditions in the lower aquifer, the availability of Fe(II) is likely too low to support a considerable contribution of iron oxidation to autotrophy. The active RubisCO-based autotrophic communities in both aquifers appeared to be dominated by a phylotype closely related to the strictly autotrophic, facultatively aerobic betaproteobacterium *Sulfuricella denitrificans* (>97% similarity at the protein level). The dominance of *S. denitrificans* may be explained by its versatile lithotrophic metabolism, as it is able to oxidize elemental sulfur and thiosulfate to sulfate aerobically or coupled to denitrification under anoxic conditions (50). A fraction of 16S rRNA reads related to *S. denitrificans* in the lower aquifer samples of 0.4 to 5.6% underlined the importance of this metabolic group within the total active microbial community. Oxidative weathering of pyrite, which is known to be associated with trochite limestone in this area, is likely to provide the energy for CO$_2$ fixation by sulfide, thiosulfate, or iron oxidizers, contributing to the microbially mediated changes to the makeup of rock and water in this aquifer system.

Aquifer-driven clustering of *cbbL* sequences was linked primarily to the distribution of reads related to ammonia-oxidizing bacteria (AOB), specifically *Nitrosomonas* sp. strain IsA73, known to harbor only RubisCO form I. The distribution of these strict aerobic microorganisms was obviously influenced by oxygen availability, since this genus was represented by a much larger *cbbL* read fraction in the oxygen-rich lower aquifer. In line with this, 16S rRNA reads related to *Nitrosomonas* sp. IsA73 accounted for 0.3 to 3.8% of the total 16S rRNA sequence reads from the lower aquifer but only <0.1% of total 16S rRNA sequence reads of the upper aquifer. These findings agree with data for qPCR targeting *amoA* genes encoding ammonia monooxygenase of AOB, which were 1 to 2 orders of magnitude more abundant in the lower than in the upper aquifer (44). Moreover, phylogenetic analysis based on *amoA* sequences revealed that AOB communities were dominated by phylotypes closely related to *Nitrosomonas ureae* (44), which is closely related to *Nitrosomonas* sp. IsA73, the dominant AOB detected in the active microbial communities in this study. Nitrification activity was also detectable in the oxygen-rich lower aquifer at 0.6 nmol NO$_3$ liter^{-1} h^{-1} (44), which is slightly higher than what has been reported for the Atlantic Ocean (73). Thus, these findings strongly indicate that nitrification plays a role in autotrophic CO$_2$ fixation in the lower aquifer.

The larger fraction of *Nitrospira*-related 16S rRNA sequence reads in the lower aquifer suggested that nitrite oxidizers related to *Nitrospira* sp. could also contribute to carbon autotrophy, using the reverse tricarboxylic acid (rTCA) cycle as the CO$_2$ fixation pathway (74). Moreover, recently reported results showed that thaumarchaeal ammonia oxidizers, which fix CO$_2$ via the 3-hydroxypropionate/4-hydroxybutyrate cycle pathway (75, 76), represented up to 65% of the archaeal population in the lower aquifer (44). Future work employing metagenomics and metatranscriptomics approaches will allow us to integrate these alternative CO$_2$ fixation pathways in a more comprehensive picture of chemolithoautotrophy in the Hainich limestone aquifers, especially with regard to the potential contribution of nitrite-oxidizing bacteria and ammonia-oxidizing *Archaea* to nitrification-based carbon autotrophy.

Estimated fraction of autotrophs within the total microbial population. Quantitative PCR targeting the *cbbM* and *cbbL* genes clearly demonstrated that a considerable fraction of the total microbial population had the genetic potential to fix CO$_2$ via the Calvin-Benson-Bassham cycle in both aquifers. Assuming a decreasing influence of surface-derived input of organic matter with increasing depth, we expected to find a greater genetic potential for autotrophy in the lower aquifer. However, in contrast to our hypotheses, we did not observe higher concentrations of TOC in the upper aquifer, nor did the abundances of RubisCO-encoding genes point to a larger fraction of autotrophs utilizing the Calvin-Benson-Bassham cycle within the total microbial population in the lower aquifer. Across the two aquifers and across all time points, the estimated fraction of putative CO$_2$-fixing bacteria within the total groundwater microbial population ranged from <1 to ~17%, which is >1 order of magnitude larger than fractions estimated for other aquifers (26), estuary sediments (77), or paddy soils (23). We cannot rule out that a certain fraction of the CO$_2$-fixing microbial population using the Calvin-Benson-Bassham cycle was overlooked, since we did not target *cbbL* genes and transcripts encoding RubisCO form IC, which has often been associated with facultative autotrophs (17). However, the low transcript-to-gene ratios observed for *cbbL* and *cbbM* suggest that only a small fraction of the CO$_2$-fixing microbial community was actually expressing their genes, indicating that rates of carbon fixation via the Calvin-Benson-Bassham cycle in the groundwater might have been low at these two time points. The genetic poten-

tial of the two aquifers to fix CO_2 via the Calvin-Benson-Bassham cycle may still be underestimated, since we did not target microbial communities attached to the rock surfaces of the aquifer, which have been shown to differ from suspended communities in their composition and metabolic capacities (78, 79).

In conclusion, our results demonstrated that pristine limestone aquifers harbor diverse and dynamic microbial communities with a considerable fraction of bacteria that are capable of autotrophic CO_2 fixation via the Calvin-Benson-Bassham cycle. Oxygen availability and distance to surface soils likely played key roles in shaping the structure of the active microbial communities, with a larger fraction of potentially soil-derived, heterotrophic microbial groups in the upper than in the lower aquifer. Most CO_2-fixing bacterial groups identified harbored both RubisCO forms I and II, indicating the potential for CO_2-fixing populations to adjust to fluctuating O_2 and CO_2 concentrations. Overall, our findings clearly point to a high genetic potential for chemo-lithoautotrophy in pristine limestone aquifers. Combined transcript analysis of 16S rRNA and RubisCO-encoding genes suggested that facultative aerobic thiosulfate oxidizers and strict aerobic ammonia oxidizers are involved in carbon fixation in the lower oxygen-rich aquifer. Further evidence of a role of ammonia oxidizers was provided by a parallel study confirming active nitrification and the presence of ammonia oxidizers based on *amoA* genes. Future studies are needed to address alternative CO_2 fixation pathways, such as the rTCA cycle and the 3-hydroxypropionate/4-hydroxybutyrate pathway, to further elucidate the role of nitrite oxidizers as well as ammonia-oxidizing *Archaea* in carbon autotrophy in these limestone aquifers.

ACKNOWLEDGMENTS

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