Communities of *Archaea* and *Bacteria* in a Subsurface Radioactive Thermal Spring in the Austrian Central Alps, and Evidence of Ammonia-Oxidizing *Crenarchaeota*[▽]

Gerhard W. Weidler,¹ Marion Dornmayr-Pfaffenhuemer,¹ Friedrich W. Gerbl,¹
Wolfgang Heinen,^{2†} and Helga Stan-Lotter^{1*}

Division of Molecular Biology, Department of Microbiology, University of Salzburg, Billrothstraße 11, A-5020 Salzburg, Austria,¹
and Department of Microbiology, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands²

Received 7 July 2006/Accepted 19 October 2006

Scanning electron microscopy revealed great morphological diversity in biofilms from several largely unexplored subterranean thermal Alpine springs, which contain radium 226 and radon 222. A culture-independent molecular analysis of microbial communities on rocks and in the water of one spring, the “Franz-Josef-Quelle” in Bad Gastein, Austria, was performed. Four hundred fifteen clones were analyzed. One hundred thirty-two sequences were affiliated with 14 bacterial operational taxonomic units (OTUs) and 283 with four archaeal OTUs. Rarefaction analysis indicated a high diversity of bacterial sequences, while archaeal sequences were less diverse. The majority of the cloned archaeal 16S rRNA gene sequences belonged to the soil-freshwater-subsurface (1.1b) crenarchaeotic group; other representatives belonged to the freshwater-wastewater-soil (1.3b) group, except one clone, which was related to a group of uncultivated *Euryarchaeota*. These findings support recent reports that *Crenarchaeota* are not restricted to high-temperature environments. Most of the bacterial sequences were related to the *Proteobacteria* (α , β , γ , and δ), *Bacteroidetes*, and *Planctomycetes*. One OTU was allied with *Nitrospina* sp. (δ -*Proteobacteria*) and three others grouped with *Nitrospira*. Statistical analyses suggested high diversity based on 16S rRNA gene analyses; the rarefaction plot of archaeal clones showed a plateau. Since *Crenarchaeota* have been implicated recently in the nitrogen cycle, the spring environment was probed for the presence of the ammonia monooxygenase subunit A (*amoA*) gene. Sequences were obtained which were related to crenarchaeotic *amoA* genes from marine and soil habitats. The data suggested that nitrification processes are occurring in the subterranean environment and that ammonia may possibly be an energy source for the resident communities.

Bad Gastein, a village in the National Park “Hohe Tauern” in the Alps near Salzburg, Austria (Fig. 1), is known for its thermal mineral springs. A cluster of 17 major springs (between 962 to 1,029 m above sea level) with temperatures up to 47°C provide about 4 to 5 million liters of thermal mineral water per day (7, 9, 15). The water of the springs is collected in such a way that nearly no surface water can mix with the warm spring water, which is distributed to the spa hotels (9). Numerous medical determinations of the influence of radium 226 and radon (mainly Rn 222), which are present in the mineral water of the Bad Gastein thermal springs, on the human organism have been made (9, 15). However, almost no information about the microbial flora of these thermal springs exists (7, 18).

One of the major springs, located at the highest altitude (1,029 m above sea level), is called “Franz-Josef-Quelle” (FJQ) (Fig. 1); it consists of a total of 27 single water discharges, issuing directly from rock fissures (18). It is located at the end of a gallery that was driven horizontally about 100 to 150 m deep into the rock. It issues from an artificial cavity from the chasms traversing the rock (7), delivering 295 m³ water per day

(according to reports from 1997-1998) with a Rn 222 quantity of 296 kBq/m³ water (7, 9, 18). Its average temperature is 45.6°C, and the pH is about 8.0. The geological environment of the spring area, consisting mainly of gneiss and crystalline slate, which are defined as primary rocks, prevents rain water from trickling into the ground. As stated by Cudrigh (7), pure and unmixed thermal mineral water possesses a mineralization degree of about 350 mg/liter and a temperature of 56°C. These properties as well as the content of tritium—which should not occur in thermal water—indicated an ~10% intermixture of the spring FJQ with colder ground or subsurface water, which was corroborated by measurements of d¹⁸O and deuterium (7). Furthermore, the subterranean water cycle was measured by the ¹⁴C method, which suggested a retention time of 3,600 to 3,800 years (7, 15). Currently, little is known of such subsurface environments and in particular about possible energy and carbon sources in such niches, but the interest in the microbiology of subterranean environments is clearly rising (14, 19, 20, 33, 46).

Caves harbor an especially interesting composition and variety of mineral-utilizing microorganisms, which may contribute to the formation of unusual netlike microstructures as described by Boston et al. and Northup et al. (4, 33). Very similar arrangements were detected by scanning electron microscopy in the springs of Bad Gastein (W. Heinen, H. P. M. Geurts, and A. M. Lauwers, unpublished data). Therefore, the spring FJQ was chosen for examination of its microbial com-

* Corresponding author. Mailing address: Division of Molecular Biology, Department of Microbiology, University of Salzburg, Billrothstraße 11, A-5020 Salzburg, Austria. Phone: 43 662 8044 7210. Fax: 43 662 8044 7209. E-mail: helga.stan-lotter@sbg.ac.at.

† Deceased 30 June 2006.

[▽] Published ahead of print on 3 November 2006.

FIG. 1. Geographical location of Bad Gastein (bottom right) and overview of the spring "Franz-Josef-Quelle," showing the area with water discharges numbered 21, 23, and 24. Water discharge 24 is above number 23 and cannot be seen in this picture. All discharges are collected in the little rock basin. The bottom left panel shows rock fissures with water leaking out (arrows).

position. The inadequacy of culture-based methods for estimation of species numbers in natural environments has been well documented (1, 35), so culture-independent techniques were applied. These included 16S rRNA gene sequence analyses and restriction fragment length polymorphism (RFLP) (20). In addition, the search for crenarchaeal ammonia oxidation-related genes (48) was applied to examine this subsurface habitat, because early results in this study revealed the presence of a large number of crenarchaeal clones and, as mentioned, the energy supply for microbial cave communities is, with some exceptions, entirely unknown.

MATERIALS AND METHODS

Sampling and site description. The samples were taken from water discharges number 21, 23, and 24, which flow into a small basin (Fig. 1). The water temperature at the point of sampling was 42.3°C with a pH value of 8.1; the air temperature was 34°C with humidity near 100%. Water samples and bacterial mats (biofilms), designated FJQF and FJQB, respectively, were taken under aseptic conditions, using sterile equipment. Water samples were collected in aluminum foil-wrapped one-liter bottles. Spatulas were used for removal of biofilm samples, which were placed into 50-ml reaction tubes. Glass slides (75 mm by 25 mm by 1 mm) were mounted into a metal rack, which held 10 slides, and were placed into the spring FJQ for up to 11 days. Following collection, the slides were placed into 50-ml reaction tubes. Samples from glass slides were

designated as FJQOT. Water samples were stored at 4°C. Biofilm samples and glass slides were stored at -70°C until further use.

Some specific data for the spring FJQ are as follows (modified from reference 7): the mineralization degree of the spring water is 366 mg/liter; the O₂ content is up to 0.3 mg/kg of water; cations (in mg/kg of water) are Na⁺ (76.3) and Ca²⁺ (19.1); anions are SO₄²⁻ (125.3), HCO₃⁻ (57.3), Cl⁻ (25.3), and F⁻ (5.4). Mn²⁺ is present as a trace element in a concentration of up to 0.03 mg/kg of water. NH₃, NH₄⁺, NO₃⁻, and NO₂⁻ are present in traces (<0.01 to 0.1 mg/kg of water).

DNA preparation from filtered water and from biofilm on glass slides. Two liters of mineral water were passed through a 0.22-μm-pore-size autoclaved membrane filter (Durapore Filters, Millipore, Bedford, MA), using a filtration unit (SM 16201/19/20; Sartorius, Vienna, Austria) with a low-pressure vacuum. Filters were kept at -70°C until further use. Glass slides that were incubated in the spring for 11 days were broken into small pieces, and DNA extraction was performed as described by Radax et al. (38).

DNA preparation from natural biofilm samples. During DNA extraction, presumptive organic or inorganic compounds, e.g., humic acids, phenolic compounds, and heavy metals, which can inhibit or decrease the sensitivity of PCR (10, 45), appeared as a brownish-reddish color, or as yellow color after DNA extraction. To eliminate these interfering compounds, the DNA extraction protocol described by T. Glenn (MUD-DNA extraction protocol; www.uga.edu/srel/DNA_Lab/protocols.htm) was used with the following modifications. About 2 g of brownish-white biofilm were centrifuged at 6,000 × g for 5 min at 4°C. After centrifugation, the watery supernatant was removed and 900 μl of TE buffer (100 mM Tris-Cl, pH 7.6, 10 mM EDTA) and 50 μl of lysozyme (10 mg/ml) were added. Samples were mixed for 2 min and incubated for 1 h at 37°C. Following boiling for 10 min, 100 μl of proteinase K (10 mg/ml) was added and samples

were incubated for 1 h at 56°C. To each sample, guanidinium thiocyanate (Sigma, Saint Louis, MO) and diatomaceous earth (Sigma), prepared as described by Glenn (www.uga.edu/srel/DNA_Lab/protocols.htm), were added. Subsequent sample preparation was done as recommended in the protocol by Glenn. DNA was further cleaned with a Gene Clean II kit (Bio-101, Carlsbad, CA) according to the instructions of the manufacturer. The yellow color was nearly completely eliminated using this protocol.

PCR and clone library construction. Several primer pairs were tested for PCR amplification of partial 16S rRNA gene fragments of the extracted community DNA from filtered water, glass slide, and biofilm samples by using a standard PCR approach. The primer pairs A21F (5' TTC CGG TTG ATC C[CT]G CCG GA) and A958R (5' [C/T]CC GGC GTT GA[A/C] TCC AAT T) for *Archaea* (11) and P797F (5' C[A/G]A [C/T][A/C] GGA TTA GAT ACC C) and E1492R (5' TAC GG[C/T] TAC CTT GTT ACG ACT T) for *Bacteria* (42) were chosen, since they yielded the highest amounts of PCR product. The reactions were performed in a total volume of 50 µl with 1.25 U of *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany). The PCR products were excised following separation by gel electrophoresis and recovered from the gel slices using a QIAquick gel extraction kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. Subsequently, two separate clone libraries were created, one for *Bacteria* and one for *Archaea*, and for each sample type (water, biofilm, and glass slides). Purified PCR products were ligated into a pGEM-T vector system (Promega, Madison, WI) and transformed into competent *Escherichia coli* JM109 cells as recommended by the manufacturer. Positive clones were cultivated overnight in selective media, and vectors were purified using a "TENS-Mini Prep" protocol (50) with slight modifications as follows. To resuspended *E. coli* pellets, 300 µl of TENS buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.1 N NaOH, 0.5% sodium dodecyl sulfate) was added and incubated for 15 min at room temperature. Following cell lysis, 150 µl of 3 M sodium acetate, pH 5.5, was mixed with the cell lysate by inverting the reaction tubes several times. Samples were incubated on ice for 15 min and centrifuged for 10 min at $17,530 \times g$ at 4°C and again for 15 min after precipitation of DNA. Samples were washed with 75% ethanol and air dried, and plasmid DNA was dissolved in 50 µl of double-distilled H₂O.

RFLP analysis and grouping of clones. Purified vectors were digested for 16 h at 37°C with DdeI (Promega) for RFLP analysis, in order to separate the clones into groups according to their restriction patterns. Because DdeI cuts at four restriction sites in the pGEM-T vector, it was decided not to amplify the inserted DNA by PCR but to digest the cloned insert together with the vector. The digests were analyzed on 3% agarose gels. Inserted fragments, which were not cut by DdeI, were amplified with *Archaea*- or *Bacteria*-specific primers by a standard PCR protocol. PCR products were digested for 16 h at 37°C with HpaII (Fermentas) and separated on 3% agarose gels. Each RFLP group was preliminarily defined as an operational taxonomic unit (OTU). As representative sequences of each OTU were available, sequences were subjected to similarity matrix analysis using Similarity Matrix version 1.1 (27) of the RDP II (28, 29), to support OTU definition and RFLP grouping, respectively. The clones were clustered into operational taxonomic units at a level of sequence similarity of >99% (30, 43) in order to quantify diversity.

Sequencing and phylogenetic analysis. At least two sequences representing each OTU group—if OTUs had more than one representative—were amplified with the primers SP6 (5' TAT TTA GGT GAC ACT ATA G) and T7 (5' TAA TAC GAC TCA CTA TAG GG). Sequences were determined by automated dideoxynucleotide methods with an ABI Prism Big-Dye Terminator cycle sequencing kit on an ABI Prism 310 genetic analyzer (Perkin-Elmer Applied Biosystems, Vienna, Austria), as recommended by the manufacturer. Sequence comparisons to identify database sequences similar to those from the clones were done using the FASTA3 (37) web interface from the European Molecular Biology Laboratory (EMBL). The potential presence of chimeric sequences was examined with the CHECK_CHIMERA program (27) available through the RDP II, release 8.1, with the chimera check program of the Bellerophon server (21), and in addition, the phylogenetic affiliations of their 5' and 3' ends were compared manually. No chimeras were recognized within archaeal sequences, but three potential chimeras were seen within bacterial sequences. Selected sequences were aligned with ClustalX (47). Subsequently the alignment was subjected to phylogenetic analysis using distance-based (22, 40) maximum parsimony and maximum likelihood methods, with programs from the phylogenetic interference package (PHYMLIP) version 3.5.1c (J. Felsenstein, University of Washington, Seattle; distributed by the author) and the ClustalX software. Trees were visualized with the Treeview PPC program version 1.6.0 (36). In addition, the significance levels of interior branch points were determined by bootstrap analysis using 1,000 data resamplings. Phylogenetic trees shown

in this work were constructed by using a neighbor-joining algorithm with ClustalX version 1.81 (40, 47).

Amplification of *amoA* (ammonia monooxygenase subunit A)-related sequences. PCR was performed with a standard touchdown approach using the primer pair amo111F and amo643R (48), which are specific for crenarchaeal ammonia monooxygenase subunit A-related genes. The primers were a gift from Christa Schleper and coworkers, University of Bergen, Norway. PCR products from filtered water, glass slides, and biofilm material were designated as F, OT, and B, respectively, and were recovered from the gel slices using a QIAquick gel extraction kit (QIAGEN) in accordance with the manufacturer's instructions. PCR products of each sample type were cloned into the pGEM-T vector, and five randomly chosen clones of each sample were sequenced. Cloning and sequencing were performed as described above.

Statistical analysis. The screening process was tested by statistical analyses to evaluate whether total diversity was covered by screening 415 clones. Two types of analyses were used. Coverage values were calculated by use of the following equation: $C = 1 - (n/N) \times 100$, where n is the number of unique OTUs and N is the total number of clones examined (see reference 39 for a discussion of terms). In addition, a rarefaction analysis was performed to determine the number of unique OTUs as a proportion of the estimated total diversity. Calculations were performed using the freeware program Analytic Rarefaction version 1.3 (S. M. Holland; www.uga.edu/strata/software/Software.html).

Scanning electron microscopy. Material for scanning electron microscopy (SEM) was taken randomly from the spring and its vicinity. A sample holder with two round 1.5-cm-diameter coverslips was placed for several days at a defined location, e.g., 2, 25, 45, and 55 cm from the rock fissures, allowing formation of a biofilm and deposition of small rock particles. The part of the coverslip that was covered by the holder was not colonized and thus served as a control. The coverslips as well as pieces of rock surface were immediately air dried and transported in a closed and dry container to the laboratory. The samples were mounted on aluminum SEM holders and coated with 1.5 nm Au/Pd (40:60) using a 208 high-resolution Cressington sputter coater (Watford, England) in order to enhance the conductance of the material and to prevent charging artifacts during scanning. Electron microscopy was carried out with a JEOL JSM-6330F field emission instrument (Tokyo, Japan) operated at 3.0 kV. This instrument is hosted by the Department of General Instrumentation of the Radboud University Nijmegen.

Nucleotide sequence accession numbers. All small-subunit rRNA gene sequences as well as the *amoA* sequences were deposited in the EMBL/EBI nucleotide sequence database under the following accession numbers: AM039528 to AM039550 for partial 16S rRNA gene sequences; AM233905 and AM260487 to AM260489 for the crenarchaeal *amoA* sequences.

RESULTS

Sampling and 16S rRNA gene analysis using RFLP. Water samples and bacterial mats (biofilms) were designated FJQF and FJQB, respectively. The microbes, which cover the surface of the submerged boulders in the spring, seemed to exist in two different forms (18), both of which were collected as samples designated FJQB: an older colonization stage of these mats adhered strongly to the rock surfaces, whereas the second form appeared as a whitish slimy substance, which probably represented the glycocalyx of diverse microorganisms, and seemed to be less firmly attached to the rocks (18). Archaeal and bacterial 16S rRNA gene fragments were amplified with primers A21F/A958R and P797F/E1492R, respectively. A total number of 415 clones was analyzed; 283 clones were of archaeal origin, and 132 clones were of bacterial origin. RFLP analysis resulted in seven archaeal groups, designated FJQGA, and 16 bacterial groups, designated FJQGB, or FJQGBL if inserts were not cut by DdeI. This was the case for five of the 16 bacterial RFLP groups, and therefore the grouping of those five was based on the digestion of the amplified insert sequence with HpaII. When representative sequences of each group became available, sequences were compared using the Similarity Matrix version 1.1 of the RDP II. This method reduced the archaeal

TABLE 1. Most similar database sequences of bacterial and archaeal 16S rRNA gene phylotypes recovered from the subsurface spring FJQ

OTU	Phylogenetic relationship			
	Phylotype	Closest database sequence (accession no.) ^a	Origin of database sequence and/or reference	% Similarity
<i>Bacteria</i>				
FJQGB1	FJQFB60	Unc. bacterium clone BCM1-7B (AY102889)	Subtropical freshwater marsh	89.42
FJQGB2	FJQFB11	Unc. bacterium clone P3OB-49 (AF414580)	Uranium mine sediment	99.42
FJQGB3	FJQFB20	Unc. bacterium clone G19 (AF407702)	Australia: Great Artesian Basin	98.23
FJQGB4	FJQBAB2	Unc. bacterium MERTZ_2CM_163 (AF424307)	Antarctic shelf sediments (5)	87.87
FJQGB5	FJQOTB2	Unc. β -proteobacterium clone ccs265 (AY133064)	TCE-contaminated site ^b	98.58
FJQGB6	FJQOTB34	Unc. bacterium clone TSAC04 (AB186880)	Bioremediation microcosm	95.64
FJQGB7	FJQBAB3	<i>M. capsulatus</i> Texas (NCIMB 11853; AJ563935)	8	94.34
FJQGB8	FJQBAB4	Unc. bacterium clone AKAU3482 (DQ125521)	Uranium-contaminated soil	97.48
FJQGB9	FJQBAB9	Unc. thermal soil bacterium YNPFPP32 (AF391976)	Extreme thermal soil	95.17
FJQGBL1	FJQFB62	Thermophilic methanotroph HB (U89299)	Underground hot spring (3)	99.16
FJQGBL2	FJQOTB21	Unc. β -proteobacterium HUuD-LB/2-3 (AB176697)	Japanese gold mine	95.06
	FJQFB23			95.11
	FJQBIB7			94.92
FJQGBL3	FJQOTB22	Unc. bacterium clone LO13.11 (AF358003)	Peat soil microcosm (31)	98.07
FJQGBL4	FJQBAB15	Unc. bacterium clone D023011 (AJ617920)	Paddy soil oxygen gradient (32)	98.32
FJQGBL5	FJQBAB22	Unc. bacterium clone 1-13 (AY548937)	ANAMMOX sludge	98.35
<i>Archaea</i>				
FJQGA1	FJQBAA5	Unc. archaeon SAGMA-2 (AB050233)	South African gold mine (46)	99.10
	FJQFA2			99.44
FJQGA2	FJQBAA3	Unc. crenarchaeote Gitt-GR-39 (AJ535120)	Uranium mill tailings	98.54
	FJQBAA20			98.65
	FJQFA13			99.32
FJQGA3	FJQOTA5	Unc. archaeon FS274-21A-03 (AY704372)	Oceanic crust	94.70
FJQGA4	FJQOTA49	Unc. archaeon pCIRA-X (AB095133)	Deep-sea hydrothermal vent site	82.15

^a Unc., uncultured.^b TCE, trichloroethene.

groups to four and bacterial groups to 14, since the similarity of some sequences was higher than 99%. These final groups were defined as one OTU each. Representative sequences of these merged groups were treated as single phylotypes included in one OTU (Table 1) and were also submitted to the EMBL/EBI nucleotide sequence database. The procedure was applied to the archaeal phylotypes FJQFA13, FJQBAA3, and FJQBAA20 (OTU FJQGA2), because these sequences were more than 99% similar to each other (data not shown), and to phylotypes FJQBAA5 and FJQFA2 (OTU FJQGA1). This procedure was also applied to bacterial phylotypes FJQOTB21, FJQFB23, and FJQBIB7 for the same reasons (FJQGBL2; Table 1).

Archaeal community composition and phylogenetic analysis. The composition of archaeal OTUs clearly showed the abundance of crenarchaeal clones in all three sample types, filtered water, biofilm (microbial mats), and glass slide, which confirmed the common suggestion that mesophilic *Crenarchaeota* represent a stable and specific component in terrestrial habitats (2, 12, 34, 41). FJQGA1 and FJQGA2 consisted of clones from all three sample types, while the dominating clones of FJQGA1 originated from filtered water and FJQGA2 stemmed from the biofilm (Table 1). The database sequence that was most similar to FJQGA1 was clone SAGMA-2 of waters from a South African gold mine (46), but the most similar database sequence of FJQGA2 was an uncultured crenarchaeon, clone Gitt-GR-39 from uranium mill tailings. This result is of interest to our examinations, since the presence of radioactivity (radium, radon, and uranium) is characteristic of the thermal springs of Bad Gastein (18). Table 2 shows that

crenarchaeal clones of FJQGA1 and FJQGA2 were the most frequently recovered archaeal groups in this habitat, representing 99.65% of all archaeal clones. A single clone, recovered from a glass slide, may have been of euryarchaeal origin, but

TABLE 2. Distribution of clones from the spring FJQ^a

OTU	No. of clones			Percentage of total
	Filter	Glass slide	Biofilm	
<i>Bacteria</i>				
FJQGB1	15			11.36
FJQGB2	1			0.76
FJQGB3	3			2.27
FJQGB4	11		3	10.61
FJQGB5	10	28		28.79
FJQGB6		4		3.03
FJQGB7			2	1.52
FJQGB8			1	0.76
FJQGB9			1	0.76
FJQGBL1	11	4		11.36
FJQGBL2	28	4	2	25.76
FJQGBL3		1		0.76
FJQGBL4			1	0.76
FJQGBL5		1	1	1.52
<i>Archaea</i>				
FJQGA1	58	32	1	32.16
FJQGA2	54	24	109	66.08
FJQGA3		4		1.41
FJQGA4		1		0.35

^a Total bacterial clones, 132; total archaeal clones, 283.

FIG. 2. Archaeal phylogenetic tree based on 16S rRNA gene sequences, including various 16S rRNA gene clones obtained from the subsurface thermal spring FJO. The tree was inferred by a neighbor-joining analysis of 772 corresponding positions of the 16S rRNA gene sequence (positions 107 to 879 of *E. coli* numbering [6]). The scale bar represents 10% nucleotide sequence difference. Four sequences from species of *Bacteria* constituted the outgroup. Symbols on the branches indicate bootstrap confidence values, as follows: ●, >90%; ○, >75%. Numbering of crenarchaeal groups was chosen in accordance to references 12, 23, and 34.

FIG. 3. Bacterial phylogenetic tree based on 16S rRNA gene sequences of members of the *Proteobacteria*, including various 16S rRNA gene clones obtained from the subsurface thermal spring FJQ. The tree was inferred by a neighbor-joining analysis of 707 corresponding positions of the 16S rRNA gene sequence. The scale bar represents 10% nucleotide sequence difference. Four sequences from species of *Archaea* constituted the outgroup. Symbols on the branches indicate bootstrap confidence values, as follows: ●, >90%; ○, >75%.

was only 82.15% similar to its nearest neighbor sequence, which was from a deep-sea hydrothermal vent site (Table 1). FJQGA3, which belonged to the freshwater-wastewater-soil (1.3b) group, appeared to be a subdominant crenarchaeal group in this habitat (Table 2; Fig. 2). Four clones, recovered from glass slides, may represent this group in the FJQ subsur-

face thermal spring but were only 94.75% similar to its nearest neighbor sequence, which was from an oceanic crust (Table 1).

Bacterial community composition and phylogenetic analysis. Bacterial 16S rRNA gene composition and abundance of OTUs showed a higher diversity in community composition than did the archaeal representatives of this subsurface envi-

FIG. 4. Rarefaction curves for total (●) as well as for archaeal (■) and bacterial (○) OTUs of 16S rRNA gene clones. The dotted lines represent 95% confidence intervals.

ronment. Several OTUs were represented by clones of only one sample type, e.g., clones obtained from filtered water appeared in FJQGB1, FJQGB2, and FJQGB3, but FJQGB2 was represented by only one clone. FJQGB7, FJQGB8, FJQGB9, and FJQGBL4 were represented exclusively by clones from bacterial mats but only by one or two clones each. The clones obtained from glass slides represented OTUs FJQGB6 and FJQGBL3, where the latter again consisted of only one clone (Table 2). The most prominent OTUs of bacterial sequences were FJQGBL2 (25% of bacterial clones) and FJQGB5 (28% of bacterial clones), followed by FJQGBL1, FJQGB4, and FJQGB1 with about 10% of all bacterial clones each (Table 2). Other groups were represented by one to four clones (Table 2). The two OTUs accounting for the largest percentages of clones were most similar to sequences recovered from a microbial mat (FJQGBL2) and from a trichloroethene-contaminated site (FJQGB5). The less dominating groups (about 10% of the abundance of bacterial clones) were composed of OTU FJQGB1, which was only 89% similar to an uncultured strain isolated from subtropical freshwater marsh (Table 1). OTU FJQGB4 had only 87% similarity to its next relative, a clone isolated from continental shelf sediments (Antarctica). FJQGBL1 (99% sequence similarity), like OTU FJQGB7 (94% similarity), was affiliated to a cluster of thermophilic methanotrophic *Bacteria* and to cultured *Methylocaldum*-*Methylococcus* strains (Table 1; Fig. 3). The less frequently recovered OTUs (one to four clones) possessed sequences which were similar to those of isolates from diverse soil-like habitats, e.g., uranium-contaminated soil, uranium mine sediment waters, ANAMMOX (anaerobic ammonium oxidation) sludges, and artificial microcosms (FJQGBL3, FJQGBL4, FJQGBL5, FJQGB2, FJQGB3, FJQGB6, and FJQGB8), as well as one extreme habitat like extremely thermal soil (FJQGB9).

Statistical analysis. Eighteen different OTUs were represented by two clone libraries, for *Bacteria* and *Archaea*, con-

sisting of 415 clones. Coverage, calculated as in reference 39, reached a value of 95.66%, indicating that nearly the total diversity was covered by the two clone libraries. However, since coverage is based on the number of unique OTUs relative to total richness, evenness is not taken into account, and thus, this value should be seen as a rough estimate of diversity. The coverage value for *Archaea* ($n = 4$; $N = 283$) was 98.58% and for *Bacteria* ($n = 14$; $N = 132$) was 89.39%. As a second approach, a rarefaction analysis was done to determine if screening of 415 clones was sufficient for an estimation of diversity in the clone libraries of the spring FJQ. The expected number of the unique OTUs was plotted against the number of clones (Fig. 4). The calculated rarefaction curve of total OTUs showed a slight tendency to saturation; the curve for archaeal OTUs showed saturation, but the one for bacterial OTUs did not reach saturation (Fig. 4), indicating that diversity was not completely covered by screening of 415 clones.

Analyses of *amoA* (ammonia monooxygenase subunit A)-related genes. Five clones recovered from each sample type were randomly selected and sequenced. Two sequences affiliated with *amoA* genes from the soil/sediment group (Fig. 5), whereas 13 clones were more similar to *amoA* genes from the marine water column or sediments. The sequences of *amoA*-related genes from *Crenarchaeota* showed similarities of 80.7% (clone F2), 78% (clone B2), and 77.2% (clone OT2) to "*Candidatus Nitrosopumilus maritimus*" (26), using FASTA Prokaryotes for the comparison. A FASTA Environmental comparison indicated 82.5% similarity of clone F2 to clone BS15.7_11 (16), 80.9% similarity of clone B2 to clone HB_A_21 (16), and 80.6% similarity of clone OT2 to clone HF130_D11 (17). All of these *amoA* genes were similar to clones of the marine water column or of marine sediments, which were described by Francis et al. (16) (Fig. 5). Clone F5, whose sequence affiliated to the soil *Crenarchaeota*, was 69.5% similar to the *amoA* gene of "*Candidatus Nitrosopumilus maritimus*," a marine *Crenarchaeon*,

FIG. 5. Phylogenetic tree of *amoA* genes cloned from the thermal spring FJQ and closely related crenarchaeal genes from the database. The tree was inferred by a neighbor-joining analysis of 546 corresponding positions. The scale bar represents 10% nucleotide sequence difference. Bacterial *amoA* from *Nitrosospira briensis* was used as the outgroup. Designation of origin from soil/sediment or water column/sediment was as suggested by Francis et al. (16). Symbols on the branches indicate bootstrap confidence values, as follows: ●, >90%; ○, >75%.

and 89.9% similar to a sequence of a terrestrial crenarchaeal *amoA* gene (DQ304894). Protein sequences were also analyzed and supported the DNA sequence comparisons. The peptide sequence of clone F5 showed an identity of 93.6% to clone OKR_C_5 (soil-sediment clone) (16) and a peptide similarity of 98.3% to an AmoA-like protein of soil *Crenarchaeota* (48). All other clones had identities between 94% and 96% and similarities of 98% to 99% to AmoA peptides of the marine water column or marine sediment origin (16).

SEM. SEM of small rock and artificial glass surfaces indicated a high diversity of morphological types of cells (Fig. 6). Figure 6A shows an overview of a microbial biofilm on pebbles, containing rod-shaped and coccoid cell types as well as spiral-shaped cells, and long thin and thick filaments. Figure 6B1 and B2 represent enlarged portions from Fig. 6A, which contain distinct spiral coiled cells and long filaments of various thicknesses. Figure 6C shows different morphotypes on a glass surface, which became attached following incubation in the spring for several days.

DISCUSSION

Subsurface environments are very often not readily accessible and provide only limited possibilities for an investigation of

the deep biosphere. However, the radioactive thermal springs in Bad Gastein, Austria, can be considered as windows to subterranean realms, since large quantities of mostly "old" thermal water ($\geq 3,000$ years) (7, 15) with very little admixture of cold surface water ($< 10\%$) are generated daily, and members of the rock-dwelling microbial communities are transported with the waters. There is little influence from environmental factors such as heavy rainfalls and thawing periods (7); thus the springs can be considered rather stable habitats, in dark surroundings (no sunlight), and not influenced by seasonal or weather-derived parameters.

The presence of archaeal and bacterial rRNA gene sequences was evident in water and biofilm samples recovered from the subsurface radioactive thermal spring FJQ, suggesting novel microbial communities in this subterranean location. Statistical analyses (Fig. 4) indicated that a large part of the archaeal diversity in this spring was likely included in the clone libraries and that further cloning would not lead to a higher number of archaeal OTUs, but more clones would most probably yield new bacterial sequences, as the diversity of bacteria was inadequately sampled. The presence of *Crenarchaeota* in this area was not too surprising since these organisms seem to appear in numerous habitats all over the world (2, 12, 34, 41).

FIG. 6. Scanning electron micrographs of natural biofilms on submerged rock surfaces in the thermal spring FJQ and microorganisms on glass surfaces, which were placed into the spring for several days. Plate A depicts an overview of a natural microbial biofilm; plates B1 and B2 show enlarged portions of plate A; plate C shows several different cell types attached to a glass surface. Different morphologies are indicated by labeled arrows (C, cocci; R, rods; S, spiral-shaped cells; F, filaments).

Crenarchaeota represent a significant fraction (up to 5%) of several prokaryotic communities (34); in some marine samples, *Crenarchaeota* constitute as much as 20% of the picoplankton (24). However, their influence on community structure and

ecology is still elusive, partly because none of these organisms, with the exception of one marine *Crenarchaeon* (26), could be cultured until now, but recent reports show the possible importance of these organisms in the nitrogen cycle of the world

FIG. 7. Bacterial phylogenetic tree based on 16S rRNA gene sequences of members of the *Nitrospirae*, *Bacteroidetes*, and *Planctomycetes*, including various 16S rRNA gene clones obtained from the subsurface thermal spring FJQ. The tree was inferred by a neighbor-joining analysis of 664 corresponding positions of the 16S rRNA gene sequence (positions 778 to 1442 of *E. coli* numbering [6]). Other details were as described in the legend to Fig. 3.

(16, 41, 48). These findings were also supported by the detection of crenarchaeal *amoA*-related genes in the spring FJQ, albeit these "soil" *Crenarchaeota* seemed to possess *amoA* genes (13 out of 15 examined clones) which were more similar to those of marine *Crenarchaeota* (Fig. 5). The dominating archaeal OTUs of the environment described here belonged to the soil-freshwater-subsurface group (1.1b); a subdominant archaeal OTU belonged to the freshwater-wastewater-soil group (1.3b) of the *Crenarchaeota* and one belonged to the

Euryarchaeota (Table 1; Fig. 2). Interestingly, all four OTUs contained isolates from glass slides, including the dominating groups, but the two subdominant OTUs were represented only by clones obtained from glass slides. This suggests that the two groups contain microorganisms that perhaps adhere to surfaces or interact with them and that sufficient DNA could only be isolated from attached cells.

The bacterial community in the spring FJQ contained two dominating groups (Table 2; Fig. 3 and 7), which comprise

9 of 14 OTUs. Sequences of these two groups affiliated with *Nitrospira* sp., *Nitrospina* sp. (δ -*Proteobacteria*) (Fig. 7), and the β -*Proteobacteria* (Fig. 3). The closest relatives of all these phenotypes were uncultivated bacteria from soils, sediments, subsurface habitats, or sludges (Table 1). Nitrification, the oxidation of ammonia to nitrate, is an essential part of the nitrogen cycle. *Nitrospirae* are aerobic chemolithotrophs, which oxidize nitrite to nitrate (33); they were isolated from seawater, where they are ubiquitous, and from soil samples. *Nitrospira moscoviensis* was isolated recently from corroded iron pipes of heating systems in Moscow, Russia (13). Also, studies of the Nullarbor caves in Australia showed clones with high similarity to *N. moscoviensis* (20). These authors described "microbial mantles" which comprise sheets or tongues of mucoid material with embedded small crystals (20). The microbial biofilms in the spring FJQ somewhat resembled this description of the microbial mantles in Nullarbor caves. In addition, the presence of *Nitrospirae* is supported by the SEM pictures: *Nitrospirae* were described as tight or loosely coiled spirals with up to 20 turns (49), a morphology which can be seen in Fig. 6A, B1, and C). The evidence for ammonia-oxidizing *Crenarchaeota* (*amoA*-related genes; Fig. 5), nitrite-oxidizing *Nitrospirae*, one *Nitrospina*-related OTU (FJQGB4), as well as one additional OTU (FJQGBL5), which was similar to a bacterial sequence from ANAMMOX-sludge, suggested that possibly a whole nitrogen cycle takes place in the FJQ spring.

Most of the other analyzed OTUs were closely similar to thermotolerant or thermophilic uncultivated *Bacteria*, with the exceptions of clone BCM1-7B, an uncultured bacterium from subtropical freshwater marsh, and clone AKAU3482, which originated from uranium-contaminated soil (Table 1). FJQGB9 was 95% similar to an isolate from extreme thermal soil, and FJQGBL1 was affiliated with a thermophilic methanotrophic strain isolated from a greenhouse heating system at a hot spring near Szentes, Hungary (3). FJQGB7 was 94% similar to *Methylococcus capsulatus* strain Texas (8). Two further dominating groups of our bacterial clone library, which were represented on glass slides, were FJQGBL2 and FJQGB5. These OTUs were similar (97 and 93%, respectively) to *Thiobacillus* species, most of which are rod shaped and 1 to 4 μ m in length, but some strains can form long filamentous aggregates (25); such morphologies also appeared in the SEM pictures (Fig. 6A and B2). Thin filaments could also stem from *Leptothrix*-related strains (44); one such sequence was detected in our clone library (OTU FJQGBL3).

The presence of diverse archaeal and bacterial sequences suggests that the thermal springs in the Central Alps near Bad Gastein and the underlying strata represent a novel and unique habitat for microbial life. The water of the springs may transport microbial communities from even deeper, more isolated and more extreme habitats to the surface. The results that we obtained in this study will provide the basis for further exploration of this subterranean environment.

ACKNOWLEDGMENTS

We thank the personnel of the water works of the community of the village Bad Gastein, especially Johann Knoll, the technical manager of the thermal springs in Bad Gastein, for help in obtaining samples. Thanks go to H. P. M. Geurts and G. J. A. Janssen, University of Nijmegen, for assistance in maintaining and operating the scanning electron microscope; to Christa Schleper and her coworkers, Univer-

sity of Bergen, for providing primers for the *amoA* gene; and to Elisabeth Pierson, University of Nijmegen, for help with the SEM pictures.

This work was partially supported by FWF project P19250-B17 and a research fellowship from the government of the Land Salzburg.

REFERENCES

- Amann, R., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**:143–169.
- Bintrim, S. B., T. J. Donohue, J. Handelsman, G. P. Roberts, and R. M. Goodman. 1997. Molecular phylogeny of *Archaea* from soil. *Proc. Natl. Acad. Sci. USA* **94**:277–282.
- Bodrossy, L., K. L. Kovacs, I. R. McDonald, and J. C. Murrell. 1999. A novel thermophilic methane-oxidising γ -*Proteobacterium*. *FEMS Microbiol. Lett.* **170**:335–341.
- Boston, P. J., M. N. Spilde, D. E. Northup, L. A. Melim, D. S. Soroka, L. G. Kleina, K. H. Lavoie, L. D. Hose, L. M. Mallory, C. N. Dahm, L. J. Crosse, and R. T. Schelble. 2001. Cave biosignature suites: microbes, minerals, and Mars. *Astrobiology* **1**:25–55.
- Bowman, J. P., and R. D. McCuaig. 2003. Biodiversity, community structural shifts, and biogeography of prokaryotes within Antarctic continental shelf sediment. *Appl. Environ. Microbiol.* **69**:2463–2483.
- Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller. 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J. Mol. Biol.* **148**:107–127.
- Cudrigh, S. F. 2002. Die Wasserwegigkeit des Gasteiner Thermalwassers, diploma thesis. University of Salzburg, Salzburg, Austria.
- Dedysh, S. N., P. Rieke, and W. Liesack. 2004. NifH and NifD phylogenies: an evolutionary basis for understanding nitrogen fixation capabilities of methanotrophic bacteria. *Microbiol. Rev.* **150**:1301–1313.
- Deetjen, P. 2006. Die wissenschaftlichen Grundlagen der Kur in Bad Gastein und Bad Hofgastein Ausgabe 2004–2006. Sem. Reports Salzburg—Austria. <http://www.zoologie.sbg.ac.at/gastein.htm>.
- de Liphay, J. R., C. Enzinger, K. Johnsen, J. Aamand, and S. J. Sorensen. 2004. Impact of DNA extraction method on bacterial community composition measured by denaturing gradient gel electrophoresis. *Soil Biol. Biochem.* **36**:1607–1614.
- DeLong, E. F. 1992. *Archaea* in coastal marine environments. *Proc. Natl. Acad. Sci. USA* **89**:5685–5689.
- DeLong, E. F. 1998. Everything in moderation: *Archaea* as 'non-extremophiles'. *Curr. Opin. Genet. Devel.* **8**:649–654.
- Ehrich, S., D. Behrens, E. Lebedeva, W. Ludwig, and E. Bock. 1995. A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, *Nitrospira moscoviensis* sp. nov. and its phylogenetic relationship. *Arch. Microbiol.* **164**:16–23.
- Engel, A. S., M. L. Porter, L. A. Stern, S. Quinlan, and P. C. Bennett. 2004. Bacterial diversity and ecosystem function of filamentous microbial mats from aphotic (cave) sulfidic springs dominated by chemolithoautotrophic "*Epsilonproteobacteria*." *FEMS Microbiol. Ecol.* **51**:31.
- Erickson, B. 2004. The therapeutic use of radon: a biomedical treatment in Europe; an "alternative" remedy in the United States, p. 600–610. *In* Proceedings of the 14th Annual Pacific Basin Nuclear Conference, Honolulu, HI, March 2004. American Nuclear Society, LaGrange Park, IL.
- Francis, C. A., K. J. Roberts, J. M. Beman, A. E. Santoro, and B. B. Oakley. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc. Natl. Acad. Sci. USA* **102**:14683–14688.
- Hallam, S. J., T. J. Mincer, C. Schleper, C. M. Preston, K. Roberts, P. M. Richardson, and E. F. DeLong. 2006. Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine *Crenarchaeota*. *PLoS Biol.* **4**:e95.
- Heinen, W., and A. M. Lauwers. 1988. Leaching of silica and uranium and other quantitative aspects of the lithobiontic colonization in a radioactive thermal spring. *Microb. Ecol.* **15**:135–149.
- Hirayama, H., K. Takai, F. Inagaki, Y. Yamato, M. Suzuki, K. H. Nealson, and K. Horikoshi. 2005. Bacterial community shift along a subsurface geothermal water stream in a Japanese gold mine. *Extremophiles* **9**:169–184.
- Holmes, A. J., N. A. Tujula, M. Holley, A. Contos, J. M. James, P. Rogers, and M. R. Gillings. 2001. Phylogenetic structure of unusual aquatic microbial formations in Nullarbor caves, Australia. *Environ. Microbiol.* **3**:256–264.
- Huber, T., G. Faulkner, and P. Hugenholtz. 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**:2317–2319.
- Jukes, T. H., and C. Cantor. 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), *Mammalian protein metabolism*, vol. 3. Academic Press, New York, NY.
- Jurgens, G., F.-O. Glockner, R. Amann, A. Saano, L. Montonen, M. Likolammi, and U. Munster. 2000. Identification of novel *Archaea* in bacterioplankton of a boreal forest lake by phylogenetic analysis and fluorescent in situ hybridization. *FEMS Microbiol. Ecol.* **34**:45–56.

24. Karner, M. B., E. F. DeLong, and D. M. Karl. 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* **409**:507–510.
25. Kuenen, J. G. 1989. Colourless sulfur bacteria, p. 1834–1871. In J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 3. The Williams & Wilkins Co., Baltimore, MD.
26. Könneke, M., A. E. Bernhard, J. R. de la Torre, C. B. Walker, J. B. Waterbury, and D. A. Stahl. 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**:543–546.
27. Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese. 1993. The ribosomal data base project. *Nucleic Acids Res.* **21**:3021–3023.
28. Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, Jr., P. R. Saxman, J. M. Stredwick, G. M. Garrity, B. Li, G. J. Olsen, S. Pramanik, T. M. Schmidt, and J. M. Tiedje. 2000. The RDP (ribosomal database project) continues. *Nucleic Acids Res.* **28**:173–174.
29. Maidak, B. L., J. R. Cole, C. T. Parker, Jr., G. M. Garrity, N. Larsen, B. Li, T. G. Lilburn, M. J. McCaughey, G. J. Olsen, R. Overbeek, S. Pramanik, T. M. Schmidt, J. M. Tiedje, and C. R. Woese. 1999. A new version of the RDP (ribosomal database project). *Nucleic Acids Res.* **27**:171–173.
30. McCaig, A. E., L. A. Glover, and J. I. Prosser. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. *Appl. Environ. Microbiol.* **65**:1721–1730.
31. Morris, S. A., S. Radajewski, T. W. Willison, and J. C. Murrell. 2002. Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. *Appl. Environ. Microbiol.* **68**:1446–1453.
32. Noll, M., D. Matthies, P. Frenzel, M. Derakshani, and W. Liesack. 2005. Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ. Microbiol.* **7**:382–395.
33. Northup, D. E., S. M. Barns, L. E. Yu, M. Spilde, R. T. Schelble, K. E. Dano, L. J. Crossey, C. A. Connolly, P. J. Boston, D. O. Natvig, and C. N. Dahm. 2003. Diverse microbial communities inhabiting ferromanganese deposits in Lechuguilla and Spider Caves. *Environ. Microbiol.* **5**:1071–1086.
34. Ochsenreiter, T., D. Selezi, A. Quaiser, L. Bonch-Osmolovskaya, and C. Schleper. 2003. Diversity and abundance of *Crenarchaeota* in terrestrial habitats studied by 16S RNA surveys and real time PCR. *Environ. Microbiol.* **5**:787–797.
35. Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. *Science* **276**:734–740.
36. Page, R. D. 1996. TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**:357–358.
37. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**:2444–2448.
38. Radax, C., C. Gruber, and H. Stan-Lotter. 2001. Novel haloarchaeal 16S rRNA gene sequences from Alpine Permo-Triassic rock salt. *Extremophiles* **5**:221–228.
39. Ravensschlag, K., K. Sahm, J. Pernthaler, and R. Amann. 1999. High bacterial diversity in permanently cold marine sediments. *Appl. Environ. Microbiol.* **65**:3982–3989.
40. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
41. Schleper, C., G. Jurgens, and M. Jonuscheit. 2005. Genomic studies of uncultivated *Archaea*. *Nat. Rev. Microbiol.* **3**:479–488.
42. Schuurman, T., R. F. de Boer, A. M. D. Kooistra-Smid, and A. A. van Zwet. 2004. Prospective study of use of PCR amplification and sequencing of 16S ribosomal DNA from cerebrospinal fluid for diagnosis of bacterial meningitis in a clinical setting. *J. Clin. Microbiol.* **42**:734–740.
43. Singleton, D. R., M. A. Furlong, S. L. Rathbun, and W. B. Whitman. 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. *Appl. Environ. Microbiol.* **67**:4374–4376.
44. Spring, S., P. Kämpfer, W. Ludwig, and K. H. Schleifer. 1996. Polyphasic characterization of the genus *Leptothrix*: new descriptions of *Leptothrix mobilis* sp. nov. and *Leptothrix dicophora* sp. nov. nom. rev. and emended description of *Leptothrix cholodnii* emend. *Syst. Appl. Microbiol.* **19**:634–643.
45. Sørensen, S. J., J. R. de Liphay, A. K. Müller, T. Barkay, L. H. Hansen, and L. D. Rasmussen. 2002. Molecular methods for assessing and manipulating the diversity of microbial populations and processes, p. 363–389. In R. G. Burns and R. P. Dick (ed.), *Enzymes in the environment*. Marcel Dekker, New York, NY.
46. Takai, K., D. P. Moser, M. DeFlaun, T. C. Onstott, and J. K. Fredrickson. 2001. Archaeal diversity in waters from deep South African gold mines. *Appl. Environ. Microbiol.* **67**:5750–5760.
47. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**:4876–4882.
48. Treusch, A. H., S. Leininger, A. Kletzin, S. C. Schuster, H.-P. Klenk, and C. Schleper. 2005. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ. Microbiol.* **7**:1985–1995.
49. Watson, S. W., E. Bock, H. Harms, H.-P. Koops, and A. B. Hooper. 1989. Nitrite-oxidizing bacteria, p. 1810–1834. In J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 3. The Williams & Wilkins Co., Baltimore, MD.
50. Zhou, C., Y. Yang, and A. Y. Jong. 1990. Mini-prep in ten minutes. *Bio-Techniques* **8**:172–173.