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Bacterial Diversity in a Deep-Subsurface Clay Environment

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The presence of bacteria in a deep clay sediment was analyzed in a 20-m-long core horizontally drilled from a mine gallery at a depth of 224 m in the Boom clay formation (Mol, Belgium). This clay deposit is the result of a marine sedimentary process that occurred 35 million years ago. Bacterial activities were estimated by measuring respiration on [^{14}C]glucose. Using the same samples, universal primers for the genes coding for eubacterial 16S rRNA were used to amplify extracted DNA. PCR products were then cloned, sequenced, and analyzed by molecular phylogeny. Our data showed a decrease in bacterial densities as a function of distance from the gallery, with few bacteria detectable by culture at more than 80 cm from the gallery wall. PCR experiments showed the presence of bacteria in all samples, and phylogenetic analyses were then used to tentatively identify these organisms. Because of low bacterial densities in deep clay samples, direct counts and enumeration of viable bacteria on diverse culture media remained negative. All experiments, both cultures and PCR, demonstrated the difficulty of analyzing samples that contain only a few poorly active bacteria as it is difficult to avoid a small contamination by active bacteria during sampling. Since the porosity of the Boom clay formation is less than the expected size of bacteria, it is possible that some of the bacteria present in this 35-million-year-old deep clay deposit derive from cells initially trapped during the sedimentation process.

With laboratory cultures, it is notoriously difficult to obtain a representative set of the true bacterial populations present in natural environments. This is even more difficult for environments characterized by extreme conditions for life. Most of the bacterial cells that are observed by microscopy may be remarkably adapted to their specific environmental conditions and may not be amenable to conventional culture conditions (6). Therefore, the specific diversity resulting from the identification of bacterial colonies that grow on culture media cannot be considered as representative of the natural populations. On the other hand, the presence of these uncultivable organisms may be revealed through the detection of their genes, particularly when using amplification procedures such as PCR (4, 13, 18, 19, 27, 28, 31, 34, 46, 57).

The initial purpose of this work was to examine a deep-subsurface clay formation for the presence of bacteria and to study the specific diversity of these organisms, because similar environments are a possible choice as repository sites for long-life nuclear wastes. Bacteria in deep-subsurface environments are commonly described (3, 7, 9, 11, 14, 15, 24, 33, 35, 37, 39, 40, 42). By way of their metabolic activities, living bacteria participate in the chemistry of the interstitial water and could possibly intervene in the migration of radionuclides through the clay formation long after the closing of the dumping site (36, 60). It is thus extremely important to know precisely if such deep and old sediments are actually free of bacteria or if some bacterial species are present either as a result of migration or because they have survived and adapted since the deposition of the sediment (29). We therefore searched for the presence of bacteria at an underground research facility, a scientific mine gallery located at a depth of 224 m in the Boom clay formation near Mol, Belgium. The deep-subsurface clay surrounding the gallery dates from about 35 million years ago, but the gallery wall is surrounded by about 40 cm of a filling material. This material was taken from the surface and deposited during building of the site for stabilization of the wall of the gallery. These two different environments were studied because they may participate either in the chemistry of interstitial water or possibly in corrosion of the storage containers, but particular attention was given to the deeper samples, which may contain bacteria adapted to long-term survival in a remote environment. The presence of such bacterial populations was analyzed by simultaneously culturing the organisms on a set of culture media and amplifying the DNA by PCR with universal primers for the genes coding for 16S rRNA (16S rDNA).

MATERIALS AND METHODS

Site of sampling. To have access to deep-subsurface samples, we used the Underground Research Facility, a mine gallery located at a depth of 224 m in the Boom clay formation near Mol, Belgium. The geological cross section is as follows: 0 to 188 m deep, Neogene sands; 188 to 280 m, Boom clay; and 280 to 450 m, alternating sand and clay formations. The Boom clay, dating from the Rupelian period (30 to 35 million years ago), is dominated by illite-smectite of marine origin; 50 to 60% of the material is smaller than 2 μm in diameter, and 40 to 45% is between 2 and 60 μm; water content is ~20% (wet weight); the median porosity is between 10 and 20 nm, and the largest pore size does not exceed 0.1 to 0.2 μm in diameter (11a); total organic content is ~3% (dry weight); the pH is 8.2 to 8.8; and the sediment temperature is approximately 20°C. The interstitial clay water is more than ~35,000 years old, consistent with its very low hydraulic conductivity (κ, ~10^{-12} m s^{-1}) (5).

Sampling conditions. Clay samples have been collected at 0.01, 0.05, 0.15, 0.3, 0.8, 1.0, 3.1, 3.2, 4.2, 5.5, 7.3, 7.6, 8.9, 11.0, 12.2, 13.5, 14.9, 15.3, 17.8, and 19.6 m along a 20-m-long core horizontally drilled from the gallery wall at a depth of 224 m (29). The drilling machine was powered by compressed air and operated without the use of drilling mud. All the usual precautions were taken to protect each sample from contamination by exogenous bacteria such as airborne bacteria, man-carried bacteria, and bacteria introduced by coring and sampling equipment. The coring equipment was thoroughly cleaned with a high-pressure washing machine, sterilized with a formalin solution (15%), rinsed with an ethanol-distilled water solution (50:50 [vol/vol], and then rinsed with sterile distilled water. This treatment was applied to all tools and parts that contacted the samples. Immediately after sampling, the cores were each transferred into a sterile plastic sheath. Transfer was performed under nitrogen flow through a

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0.2-μm-pore-size filter, and then the sheath was sealed to prevent oxygen entry. Samples were then sent by airplane to laboratories for subsampling. To collect subsamples, we selected core sections that had no visible fissures. Cores were 10 cm in diameter, and the outer part was aseptically eliminated so that subsamples were collected (about every 20 cm) from only the uncontaminated inner portion. All subsampling operations were accomplished in a sterile anoxic glove box to reduce bacterial contamination. Subsamples were then either treated immediately or frozen in liquid nitrogen and then stored at −80°C until further analysis by PCR.

We also collected interstitial water by way of three piezometer filters extending horizontally from the gallery wall at a depth of 224 m. These piezometers collected interstitial pore water samples at increasing distances from the gallery wall (3, 7, and 15 m). This equipment was sterilized by autoclaving or flaming when possible. The initial water was not used in order to prevent contamination by airborne bacteria. Water was then collected in sterilized glass flasks that were kept in a refrigerator (4°C). Because of the low water flow rate (20 to 50 ml/day), a 10- to 60-day period was necessary to obtain the 1 to 2 liters required to carry out the whole set of microbial ecology studies.

Bacterial counts. (i) Direct counts. Each sample of clay was diluted 10-fold in a filtered (0.2-μm pore size) mineral salts solution (MSS) simulating the mineral salts composition of the Boom formation water ($MgCl_2 \cdot 6H_2O$, 22 mg; KCl, 25 mg; Na_2SO_4, 30 mg; NaCl, 40 mg; $NaHCO_3$, 1,170 mg; H_2O, 1,000 ml) supplemented with formaldehyde (4% final concentration). To separate bacteria from clay particles, this slurry was sonicated for 5 min in a Bransonic 221 ultrasonic cleaner (48 KHz, 50 W) before being processed further. Moderate sonication or strong mechanical agitation proved to be necessary for dispersal of clay particles (55). Each slurry was then diluted again 100- or 1,000-fold (final dilution, 10^{-3} or 10^{-4}). These high dilutions were necessary because of the fine granulometry and low density of clay particles. At lower dilutions, the coat of clay particles was too thick for examination by microscopy. A 1-ml aliquot of each of these diluted slurries was filtered on a 0.2-μm-pore-size black Nuclepore polycarbonate filter, stained for 3 min with acridine orange (20) or DAPI (4′,6-diamidino-2-phenylindole) (41), and examined by epifluorescence microscopy. Clay smears were also dehydrated by the critical-point technique, gold plated, and examined with a JEOL 35 scanning electron microscope.

(ii) Counts of viable bacteria. Slurries were prepared by diluting each sample 10-fold in MSS, sonicated as described above, and inoculated into a set of 13 culture media adapted for growth of the most frequently described physiological groups of subsurface bacteria: aerobes or facultatively heterotrophic anaerobes (Bacto Peptone, 5 g; Bacto Agar, 15 g; MSS, 1,000 ml), heterotrophic anaerobes (same composition as above, supplemented with the vitamin, salt, and oligoelement solutions of Pfennig et al. [38] and incubated under anaerobic conditions), macroaerophiles (17), sulfate-reducing bacteria (38), hydrogen-utilizing methanogens and aceticlastic methanogens (17), *Sphaerotilus* spp. (1), *Leptothrix* spp. (32), *Gallionella* spp. (25), *Thiobacillus* spp. (56), anaerobic thiobacilli (52), ammonium-oxidizing bacteria (58), and nitrite-oxidizing bacteria (59). All culture media and methods were as described in the references cited except that MSS was used as the diluent. For each sample, a dilution series was prepared up to 10^6 in an anaerobic glove box. These dilution series were used to inoculate each of the 13 media; solid media (four plates per dilution) were inoculated up to the 10^3 dilution, and liquid media (three tubes per dilution) were inoculated up to the 10^6 dilution. All cultures (13 different culture media for each of the 20 clay samples) were incubated at in situ temperature (20°C) for periods ranging from 15 days for the Bacto Peptone agar to 90 days for all liquid culture media.

Measurements of bacterial activity. A solution of D-(U-^{14}C)glucose with a specific activity of 10.6 GBq mmol^{-1} (Amersham Corp.) was added to 1,000-ml clay slurries (diluted 1:10 [wt/vol] in MSS) to a final concentration of 5 μg of C liter^{-1}. Vessels were sealed under nitrogen flow and sampled under the same conditions by the Hungate method for anaerobes. Measurements were done in duplicate. These labeled samples were incubated in a sterile nitrogen atmosphere at 20°C (the in situ temperature) for periods ranging from 3 to 30 h. Subsamples were sequentially collected while the culture vessels were maintained under a nitrogen flow. The subsamples were acidified (6 N HCl) in a bubbling vessel. Released CO_2 was flushed by blowing N_2 (100 ml min^{-1}) for 30 min. Carbon dioxide was trapped in two serial scintillation vials containing a trapping cocktail of ethanolamine-methanol-ACS (Aqueous Counting Scintillant [Amersham]) (1:1:7 [vol/vol/vol]). Labelled carbon was counted in a Packard 1600 TR scintillation counter. Data were corrected using data for controls consisting of similar samples treated with formaldehyde to kill all viable bacteria prior to ^{14}C addition. Pore water samples were labeled with the same molecule under the same conditions, with a final concentration of 0.5 μg of C liter^{-1}. Bacterial respiration was measured as described above. Bacterial assimilation was estimated by harvesting bacterial cells on a 0.2-μm-pore-size Nuclepore filter. ^{14}C incorporated into the cells was assessed for pore water samples and was measured by liquid scintillation counting. The background signal was measured as incorporation in formaldehyde-treated samples.

Bacterial DNA extraction. Methods used for extracting bacterial DNA from solid samples can be divided into two categories: (i) direct lysis of bacterial cells followed by DNA extraction and purification (4, 47, 50, 54) and (ii) a two-step procedure involving the removal of bacterial cells from the sample followed by DNA extraction from isolated cells (22, 50, 53). Preliminary experiments undertaken with clay samples inoculated with known amounts of reference bacteria showed that a much better recovery resulted when a direct method, with extraction and lysis by physical means (i.e., using beads and a bead beater), was used, in agreement with previously published data (26, 50). The external layer was removed from each clay sample, in order to avoid any contamination introduced during coring and packaging. One gram of clay was then introduced into a 30-ml Falcon tube and vortexed for 5 min with 2 ml of phosphate buffer (0.1 M Na_3PO_4, pH 8) until the clay was completely suspended. One milliliter of lysis buffer (0.1 M NaCl; 0.5 M Tris, pH 8; 10% sodium dodecyl sulfate [SDS]) was then added, as well as 1 ml of 0.5-mm-diameter glass beads. The tube was then introduced into a bead beater apparatus for 10 min. Large particles were removed by a 5-min centrifugation at 16,000 rpm (39,000 × *g*) at room temperature. The supernatant was retrieved, while the pellet was extracted a second time as described above. Both supernatants were pooled and extracted twice with 1 volume of a phenol-chloroform mixture (1:1 [vol/vol]) and then once with 1 volume of chloroform. RNA molecules were then removed from the aqueous phase by incubation at 37°C for 10 min with 20 μl of RNAse (RNace-it; Promega).

Interstitial water collected by the piezometers also contained some clay particles. Clay particles were separated by centrifugation, and the pellet was treated in the same manner as the clay samples. The aqueous phase was boiled for 5 min in 0.15 M NaCl–0.1 M EDTA–1.25% SDS and then cooled on ice for 15 min. Both phases were pooled and extracted as described above.

Purification of bacterial DNA. Although good recovery of DNA from clay samples resulted when this procedure was used, the DNA extracted could not be amplified as such by PCR, most likely because of the presence of high concentrations of humic acids. Experiments were then undertaken to purify DNA away from humic acids; they involved differential precipitations, separation by the use of organic solvents, purification in polyvinylpyrrolidine (PVP)-agarose gels (61), and binding to selective ligands (23, 50, 53). These experiments showed better recovery and reproducibility with the use of an affinity column that specifically binds DNA and not humic acids. The use of agarose gels with PVP (Sigma; molecular weight, 360,000) was a relatively efficient means of removing humic acids, but PVP proved to be an inhibitor of the *Taq* DNA polymerases, and its use would have required further purification because of the high yields required by the low amount of template DNA. For this study, the aqueous phase obtained after the extraction step was therefore applied to Elutip-d columns (Schleicher & Schuell); the columns were then washed with 0.2 M NaCl–1mM EDTA–20 mM Tris (pH 7.4), and DNA was eluted with 400 μl of buffer (20 mM Tris, pH 7.5; 1 M NaCl) and incubated overnight at −20°C after addition of 2 volumes of ethanol containing 20 μg of glycogen and 0.3 M sodium acetate. After a 1-h centrifugation at 4°C and 13,500 rpm (12,000 × *g*), the pellet was dried and dissolved in 20 μl of distilled water.

rDNA amplification. Standard PCR conditions routinely used in our laboratory to amplify seawater samples or samples containing pathogenic bacteria failed to provide strongly positive PCR signals with clay samples, even after careful separation of humic acids. Experiments were therefore undertaken with clay samples inoculated with known amounts of reference bacteria in order to determine the best conditions for amplification. During these experiments, it appeared that in addition to the salt concentration and the temperature of hybridization, the thermoresistant DNA polymerase used was also a critical factor. A number of different enzymes (seven) from different suppliers were tested to determine which had the highest efficiency. The Goldstar *Taq* DNA polymerase (Eurogentec, Angers, France) was finally selected because it provided the best level of detection, as indicated by the presence of visible 16S rDNA PCR products in agarose gels, during amplifications with decreasing amounts of template (*Escherichia coli* or *Mycobacterium smegmatis*). For each amplification, 2.5 μl of purified DNA was used to amplify the small-subunit rRNA genes with two universal eubacterial primers, which corresponded to positions 8 to 27 and 906 to 925 of the *E. coli* small-subunit rDNA sequence. Amplifications were performed with 250 nM each deoxynucleotide, 2.5 mM $MgCl_2$, 0.4 U of polymerase, and the provided polymerase buffer [1× final concentration: 75 mM Tris HCl, pH 9.0; 20 mM $(NH_4)_2SO_4$; 0.1% (wt/vol) Tween 20] in a final volume of 50 μl. The denaturation step consisted of heating the reaction mixture at 95°C for 45 s; this was followed by an annealing step (53°C for 90 s) and an extension step (72°C for 60 s), except for the first cycle, in which the denaturation step lasted 5 min. The thermal profile consisted of 30 cycles of denaturation, annealing, and extension. A final extension was carried out at 72°C for 5 min. This amplification reaction produced DNA molecules consisting of about 900 bp. This is a shorter length than we usually use (i.e., about 1,450 bp), but it proved to provide a higher degree of amplification. The PCR products were analyzed on a 1% low-melting-point agarose gel stained with ethidium bromide and visualized by UV transillumination. Gels included a molecular weight standard for determination of molecular weights and approximative quantification of the PCR yield.

Analysis of PCR products. A subset of each PCR product was cloned in plasmids by using the TA cloning kit (Invitrogen Corporation). These plasmids were used to transform competent *E. coli* cells. Cells were plated on antibiotic-containing plates, and transformed colonies were selected as usual according to their white color. A number of transformants were randomly picked and grown in liquid medium for preparation of plasmids. Sequences were obtained by using T7 DNA polymerase as previously published (44).

TABLE 1. Taxonomic identification[a]

OTU	Clone	Clade[b]	Related taxon[c]	Contaminant
1	Bacterium 7m-04	*Proteobacteria* beta	<>*Azoarcus* and *Rhodocyclus*	No
	Bacterium 7m-06	*Proteobacteria* beta	<>*Azoarcus* and *Rhodocyclus*	No
	Bacterium 7m-03	*Proteobacteria* beta	<>*Azoarcus* and *Rhodocyclus*	No
	Bacterium 7m-05	*Proteobacteria* beta	<>*Azoarcus* and *Rhodocyclus*	No
	Bacterium 7m-23	*Proteobacteria* beta	<>*Azoarcus* and *Rhodocyclus*	No
	Bacterium 7m-10	*Proteobacteria* beta	<>*Azoarcus* and *Rhodocyclus*	No
	Bacterium 7m-16	*Proteobacteria* beta	<>*Azoarcus* and *Rhodocyclus*	No
	Bacterium 7m-25	*Proteobacteria* beta	<>*Azoarcus* and *Rhodocyclus*	No
	Bacterium 7m-22	*Proteobacteria* beta	<>*Azoarcus* and *Rhodocyclus*	No
2	Bacterium 3m-01	*Proteobacteria* gamma	<*Acinetobacter*>	No
3	Bacterium 7m-21	*Proteobacteria* gamma	<*Pseudoalteromonas*>	No
4	Bacterium 7m-24	*Proteobacteria* gamma	<*Pseudomonas*>	No
5	Bacterium 3m-02	FLGC	<>*Desulfotomaculum*	No
	Bacterium 7m-14	FLGC	<*Desulfotomaculum*>	No
	Bacterium 7m-02	FLGC	<*Desulfotomaculum*>	No
6	Bacterium 7m-13	FLGC	<*Desulfotomaculum*>	No
7	Bacterium 3m-05	FLGC	<*Desulfotomaculum*>	No
	Bacterium 3m-11	FLGC	<*Desulfotomaculum*>	No
	Bacterium 7m-12	FLGC	<*Desulfotomaculum*>	No
	Bacterium 7m-15	FLGC	<*Desulfotomaculum*>	No
8	Bacterium 3m-03	FLGC	<*Streptococcus*>	Yes
9	Bacterium 3m-04	FLGC	<*Streptococcus*>	Yes
	Bacterium 3m-07	FLGC	<*Streptococcus*>	Yes
	Bacterium 7m-08	FLGC	<*Streptococcus*>	Yes
	Bacterium 3m-12	FLGC	<*Streptococcus*>	Yes
10	Bacterium 7m-11	FLGC	<>*Carnobacterium*	No
11	Bacterium 7m-07	FLGC	<*Staphylococcus*>	Yes
12	Bacterium 3m-09	FLGC	<*Clostridium*>	No
	Bacterium 7m-01	FLGC	<*Clostridium*>	No
13	Bacterium 3m-06	FHGC	<*Propionibacterium*>	No
14	Bacterium 3m-08	FHGC	<*Propionibacterium*>	No
	Bacterium 3m-14	FHGC	<*Propionibacterium*>	No

[a] These identifications were obtained by using molecular phylogenies to compare the 16S rDNA sequences of the clones to a database of 3,400 eubacterial sequences.
[b] Three eubacterial taxa were represented: *Proteobacteria*, *Firmicuta* high G+C (FHGC), and *Firmicuta* low G+C (FLGC).
[c] <> genus, the OTU was closely related to this genus but could not be included in it; <genus>, the OTU was included in this genus.

Phylogenetic analysis and alignment: general procedure. Partial sequences used to determine the identity of each clone extended from position 240 to position 510 in reference to the *E. coli* small-subunit rDNA sequence. Such a 250-base-long domain is adequate for determining bacterial diversity (18) because an estimation of diversity does not require a precise taxonomic determination but rather the recognition of different operational taxonomic units (OTUs). Indeed, our partial sequences over a length of about 200 to 250 nucleotides included a variable domain that was sufficient to distinguish different OTUs and was adequate to place each sequence roughly in a phylogenetic tree. These short sequences were, however, not adequate to obtain either a definitive identification down to the species level or a good placement at the phylum level, that is, to resolve precisely all phylogenetic relationships. Longer sequences of the 16S rDNA gene have therefore been obtained for representatives of each OTU (i.e., positions 99 to 925), thus providing much more phylogenetic information (a length of about 800 nucleotides).

The phylogenetic data described below were obtained by (i) using successive alignment and phylogeny procedures and (ii) reinvestigating deep branching patterns after close relationships were determined. In each phylogenetic analysis, we restricted the comparisons to nucleotide positions that were definitely aligned. Some analyses were performed several times, with or without small domains that could have reached the point of saturation with mutations. Although this approach was probably not as efficient as carefully weighting each position independently, it was easier to use and was probably a reasonable compromise considering the possible problems with crossing over that sometimes affect rRNA sequences (49). For each phylogenetic analysis, in order to keep computation times within reasonable limits, it was not possible to include all representatives of outgroups and ingroups. This problem was alleviated by performing multiple analyses with different outgroups and different ingroups. Finally, we excluded sequences that were distant outgroups in order to allow better resolution of relationships among closely related species (48). All sequence alignments and species selections were done with computer programs developed by us, which are available from R. Christen upon request.

Trees were obtained by using a neighbor-joining algorithm like that developed by Saitou and Nei (45). The program was rewritten to include inputs and outputs compatible with the ribosomal database and other programs developed in our laboratory (running on 386-compatible personal computers and above). Finally, all trees were plotted by using a Macintosh computer and a program (njplot) developed by M. Gouy (Unité de Recherche Associée 243, Centre National de la Recherche Scientifique, Université Claude Bernard, Villeurbanne, France) that allows transformation of a formal tree representation (Newick's format) into MacDraw drawings.

Nucleotide sequence accession numbers. All sequences, labelled as in Table 1, have been submitted to the EMBL databank under accession numbers Z73430 to Z73461.

RESULTS

Bacterial density as a function of the distance from the gallery wall. All attempts to count bacteria by epifluorescence microscopy after labelling with the fluorescent dye acridine orange (20) or DAPI (41) or after hybridization with a fluorescently labelled nucleic acid probe complementary to a conserved domain of the 16S rRNA (43) were unsuccessful, as were all attempts to observe bacterial cells on palladium-plated clay smears, at any slurry dilution, by scanning microscopy.

Large numbers of heterotrophic bacteria (10^5 CFU ml^{-1}) were observed in samples obtained within a few centimeters of the gallery wall (Fig. 1). As the distance from the gallery wall increased, the microbial density decreased, and viable counts appeared to be limited to a few CFU per milliliter of clay at distances greater than 80 cm (Fig. 1). In all samples studied, the highest viable counts were recorded in aerobic conditions. Anaerobic bacteria were always either poorly represented or under the detection limit ($\sim$10 cells ml^{-1}). Each of the 13 enrichment culture media that were seeded with deep-clay

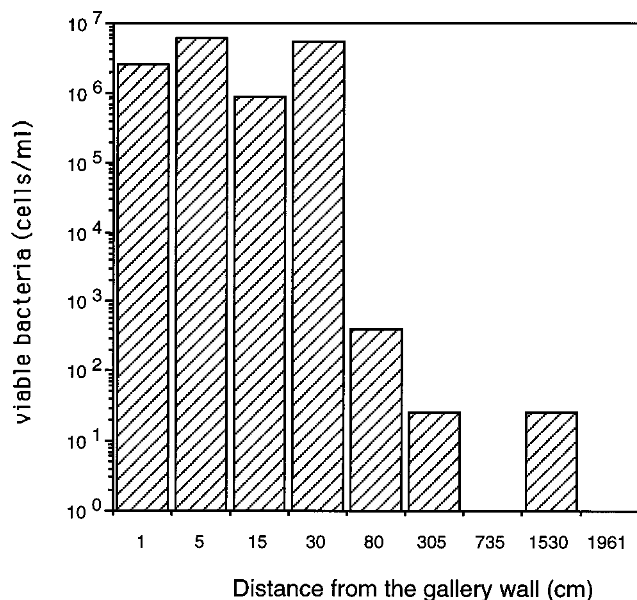

FIG. 1. Viable bacteria in clay as a function of depth from the gallery wall. CFU in samples of clay retrieved at increasing distances from the gallery wall were measured as described in Materials and Methods. There was a sudden decrease in amounts of bacteria around 80 cm, with few or no bacteria at depths greater than 3 m (all colonies retrieved at these depths were identified as likely contaminants; see the text for details).

samples to enumerate diverse, physiologically specialized microflora showed no sign of endogenous bacteria even after incubation periods prolonged up to 3 months. Most-probable-number determinations in Boom clay extracts diluted with MSS were also negative.

Bacterial mineralization of ^{14}C-labeled glucose. Bacterial respiration measurements showed a gradient decreasing from 92 nmol of CO_2 liter^{-1} h^{-1} for samples retrieved from beneath the gallery wall to 0.2 nmol of CO_2 liter^{-1} h^{-1} at a depth of 80 cm (Fig. 2). In samples collected at greater depths in the clay formation, the glucose respiration rate was very low, i.e., less than 2 pmol of CO_2 liter^{-1} h^{-1}.

Presence of bacteria revealed by positive PCR amplifications. Four samples of clay, collected at increasing distances from the gallery wall (5 cm, 80 cm, 3 m, and 7 m), and two samples of interstitial water, collected by means of two deep piezometers located 3 and 7 m from the gallery wall, were tested for the presence of bacteria by PCR amplifications with universal primers for eubacterial 16S rDNA. These assays clearly showed that bacterial DNA was present in each sample. Since extractions and PCR experiments had been conducted under exactly the same conditions for all of the samples, a smaller amount of PCR product may be indicative of a smaller abundance of bacteria. Our data (not shown) suggested that there was a greater abundance of bacteria in the vicinity of the gallery wall and that bacterial densities decreased at a distance of about 80 cm from the gallery wall.

Identification of bacteria by cloning of PCR products. Two samples, collected 3 and 7 m from the gallery wall, were selected for a more detailed analysis of bacterial diversity by cloning. Respectively, 12 and 20 clones from these two samples were randomly chosen and analyzed. Partial rDNA sequences were first obtained for a domain of about 250 nucleotides near the 5′ end of each of the sequences that was chosen because it comprises a part that is different for different species (10) and a more conserved domain that allows rapid, although approximative, phylogenetic analyses of more distant relationships. The existence of different 16S rDNA sequences cannot be considered as definitive proof that two organisms are different species. As a consequence, in this study, two sequences that had more than 2.5% divergence over the domain analyzed were identified as two different OTUs. All partial sequences were aligned together and in reference to a database containing about 3,400 eubacterial 16S rDNA sequences that were already aligned. Sequence comparisons and phylogenetic analyses (Fig. 3) showed that they belonged to 14 different OTUs. Six OTUs were present more than once, independently of their spatial localization: OTU 1 (nine clones), OTU 3 (two clones), OTU 5 (three clones), OTU 7 (four clones), OTU 9 (four clones), and OTU 14 (two clones). Four of the OTUs were detected in both samples: OTU 5 (one clone at 3 m and two clones at 7 m), OTU 7 (two clones at 3 m and two clones at 7 m), OTU 9 (three clones at 3 m and one clone at 7 m), and OTU 12 (one clone at 3 m and one clone at 7 m). OTU 1 was not retrieved in the two samples, despite the fact that it was the most abundant of all the OTUs obtained.

The phylogenetic analysis undertaken with the 250-nucleotide stretch did not allow the construction of a true phylogenetic tree (see Fig. 1), as each phylum was not properly delineated. These short sequences could be sufficient to retrieve similar sequences by a homology search, but a proper phylogenetic analysis is conducted with confidence only when a longer sequence is used. For representatives of each of these 14 OTUs (Fig. 3), the entire sequence of the PCR product was therefore obtained, and each OTU was identified by specific phylogenetic analyses. Some OTUs could be classified to well-known genera, while other OTUs were deeper branches that could not be clearly classified to any single genus. The final results are indicated in Table 1. When an identification at the genus level could not be done, each sequence was nevertheless included in a clade of higher rank (Table 1).

Identification of contaminants. Aerobic cultures seeded

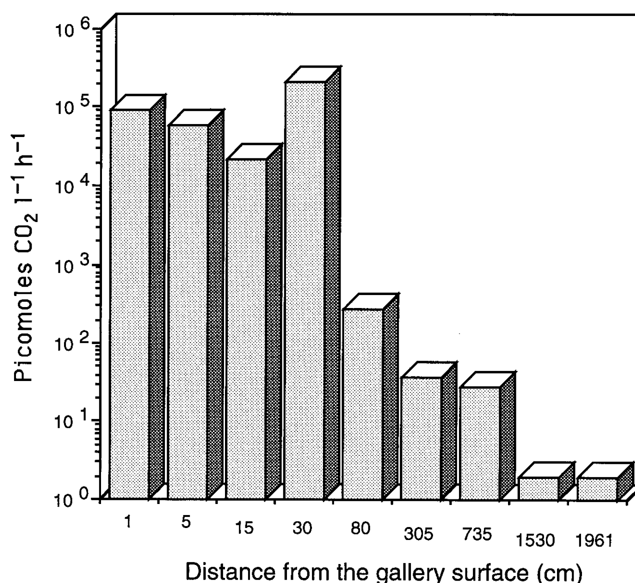

FIG. 2. Metabolization of [^{14}C]glucose by clay microflora as a function of the distance from the gallery wall. Metabolization of glucose showed the same pattern as the detection of CFU (Fig. 1). A drastic decrease in activity was observed near 80 cm, and bacterial activity was near the detection limit in the samples collected at greater depths.

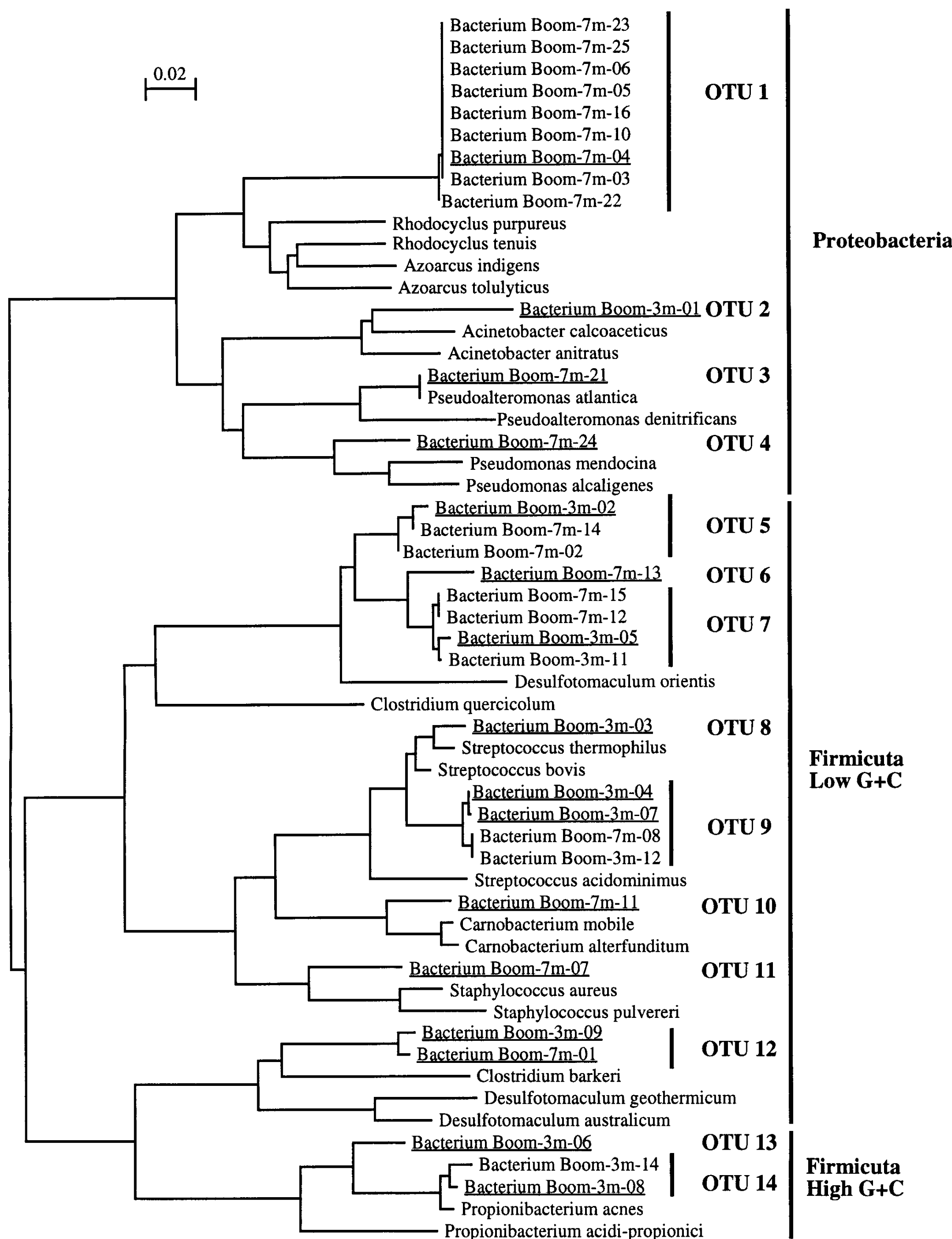

FIG. 3. Neighbor-joining analysis of the 32 clones sampled. For each sequence, the first number refers to the depth at which the sample was obtained and the second number refers to the ordinal number of the clone during the cloning procedure. Thick vertical lines are used to delineate groups of sequences that had less than 2.5% sequence differences. Underlined clones have been selected for obtaining a longer 16S rDNA sequence that allowed the taxonomic identifications shown on the right. Currently available 16S rDNA sequences of the most closely related bacteria have also been included.

with samples containing very few bacteria are prone to contamination. The few clones isolated from clay under these conditions may therefore be contaminants introduced during the culture procedure; they were therefore submitted to a phenotypic identification procedure as previously described when putative contaminants originating from the gallery itself had been sampled and identified (8). This identification procedure revealed that all of the clones belonged to the genera *Staphylococcus*, *Streptococcus*, *Microbacillus*, and *Bacillus*. Since it is very common to obtain a few similar contaminants even under strict aseptic conditions, we concluded that these clones were more likely contaminants introduced during culturing than true endogenous bacteria.

Since the detection of bacteria by PCR is a very sensitive procedure, it is also open to contamination. Two likely sources of contamination are (i) carryover of PCR products originating from other PCR experiments in the laboratory and (ii) introduction of exogenous bacteria during sampling. Contamination

by carryover was prevented by using filter-containing tips for pipetting and disposable plasticware throughout all experimentation. We can probably rule out this source of contamination because all control PCR experiments performed without clay sample DNA were negative and because none of the sequences obtained corresponded to any sequence from other experimental work in our laboratory. Contamination by exogenous bacteria during sampling was a serious concern because these deep-subsurface samples, severely confined for a long period of time, would contain very few if any bacteria. Therefore, any bacteria introduced during sample processing would become apparent, even if the number of contaminating cells was extremely small. All samplings had been done under conditions that were as aseptic as possible; in particular, all coring tools had been decontaminated before use, as detailed in Materials and Methods. However, strictly aseptic working conditions were extremely difficult to achieve, considering that a 20-m-long borehole had to be introduced at a depth of 224 m. Therefore, the grease used for the coring tools, the surrounding air, and the surface of the gallery wall were sampled, and bacteria were isolated from each of these samples and identified. A total of 200 bacterial colonies were thus isolated, and the rRNA genes of 74 of these organisms have been sequenced (8). These 16S rDNA sequences have been included in the database of 16S sequences that is used for checking bacterial identification. None of the sequences obtained by PCR and cloning from the deep clay was identical to or clustered near these likely contaminants. However, a number of OTUs had sequences that were nearly identical (less than 1% difference) to those of bacteria belonging to genera usually viewed as likely contaminants (see Table 1).

DISCUSSION

All of our attempts to count bacteria in clay by epifluorescence microscopy or scanning electron microscopy failed. These results confirm that it is extremely difficult to count bacteria in clay samples by microscopy, either with fluorescent dyes or with DNA probes. In fact, we never clearly identified bacteria. Indeed, to avoid the presence of an abundance of clay particles to the Nuclepore filter surface, it was necessary to dilute the samples to an extent that made it impossible to detect bacterial cells at a density lower than 10^7 or 10^8 cells ml^{-1}. Such a high detection limit therefore alleviates the significance of any negative count obtained by microscopy, especially when very few cells are expected in samples composed of finely sized particles.

Viable counts as well as measurements of microbial activity showed that bacterial densities and bacterial activities decreased as a function of depth from the gallery wall, i.e., as samples became representative of the undisturbed clay. Bacterial densities and metabolic activities similar to those of surface ground exist near the gallery wall (to a distance of 80 cm), probably in part because this layer is composed of a filling material originating from the surface and deposited at the opening of the site for stabilization of the wall of the gallery. By contrast, the very low counts (a few cells or less per milliliter) and very low respiration rates (2 pmol of CO_2 liter^{-1} h^{-1}) detected in the undisturbed-clay samples, collected more than 1 m from the gallery wall, are consistent with the extreme conditions for life due to the severe confinement conditions (39, 40). The extrapolation of our experimental data suggests an overall organic carbon mineralization of 0.6 mol of C liter^{-1} over a 35-million-year period. It is likely that the addition of glucose and experimental stress lead to overestimation of microbial activities by radiotracer techniques (39), considering that glucose is only a surrogate for sedimentary organic matter and that CO_2 production measurements in slurries are only potential measurements since this metabolism is not possible in deep clay. The metabolism thus measured cannot be presumed to be equivalent to what cells may have been doing in situ, but the overall organic carbon mineralization obtained is compatible with the organic carbon pool still presently observed in the Boom clay formation (3% [dry weight]) and would confer a metabolic role to these bacterial populations on the geologic time scale. On the other hand, the activity in the backfill material (90 nmol of CO_2 liter^{-1} h^{-1}) can be sustained for the short period during which this gallery has been managed. Over a 12-year period (the life of the gallery), such bacterial activity would require (with an average yield of 50%) an overall uptake of 19 mmol of organic C liter^{-1} (~228 mg of organic C liter^{-1}). Such an active metabolism could not be maintained over a 35-million-year period because it would require an overall uptake of 55×10^3 mol of organic C liter^{-1}. This estimate (~662 kg of organic C liter^{-1} of clay) is clearly unrealistic.

In deep clay, bacteria are trapped in a lattice of extremely small porosity; their movements must be extremely reduced, they must have adapted to a slow rate of cell division over time, they must have reduced activity because nutrients are available only through diffusion, and their density must be low. These conditions may explain why these bacteria have not been amenable to laboratory culture in nutrient-rich media. As a result, the very few colonies that were obtained under culture conditions are likely contaminants (and have been identified as such), which are very difficult to avoid when samples containing very few, slow-growing bacteria are collected with tools that are difficult to sterilize and under difficult working conditions, such as in a mine gallery (7, 36).

PCR experiments showed the presence of bacteria in all samples studied, and smaller amounts of PCR products suggested reduced numbers of bacteria in the undisturbed-clay samples. This result is consistent with the decreasing metabolic activities measured by the use of [^{14}C]glucose. It could therefore be concluded that molecular methods are far superior to cultures for the analysis of remote environments poorly colonized by bacteria that are difficult to isolate in the laboratory. The PCR method is, however, not devoid of artifacts. This method would, for example, also amplify genes from dead organisms and even from free DNA. In the case of the Boom clay site, the rather high water content, as well as the temperature of the clay (20°C), most likely precludes DNA molecules from remaining sufficiently intact, since their deposition 35 million years ago or during the amount of time required for diffusion with interstitial water (35,000 years), for successful amplification of a fragment of almost 1 kb.

Another problem might be due to contamination during sampling under difficult conditions. For this reason, we have sampled the surrounding environment for likely contaminants (8), and none of the cloned sequences were related to bacteria that were thriving in the gallery or on the coring tools. However, comparisons with the database of known 16S rDNA sequences revealed that some of the sequences were closely related to sequences of already-known bacterial species, as there was less than 1% difference between the sequences of some OTUs and the most closely related sequences in the database (Table 1). Since these bacteria were *Staphylococcus* or *Streptococcus* spp. of human origin, it is likely that they are contaminants introduced at one point during sampling, although, as for *Streptomyces* spp., they may occur in the environment (30). It is remarkable that such contaminants have never been obtained in other PCR and cloning experiments

done in our laboratory. Other samples that we have handled contained endogenous bacterial densities that were greater by at least 1 order of magnitude than that contained in the clay sediments. Therefore, taking into account the particularly difficult sampling conditions and the low bacterial densities of the samples, it is not unreasonable that even if very few contaminants have been introduced during the successive steps of handling, they were retrieved by PCR. One might therefore suggest that all sequences that were not related to any sequences in the database were also introduced during handling and that they are not of endogenous origin. This is always a difficult question, one which perhaps can be answered for some organisms. Even if some OTUs belong to deep branches not related to any known cultivated bacteria, these organisms could be as yet unknown surface organisms introduced during sample handling. For example, OTU 1, which was found in large amounts in one sample, is related to bacteria (*Azoarcus* and *Rhodocyclus* spp.) usually found in the environment (2, 21) and could be a contaminant introduced during coring; such bacteria have not, however, been isolated from the gallery environment (8). The situation is perhaps clearer for OTUs 5, 6, and 7, which are strongly related to sulfate-reducing bacteria (*Desulfotomaculum* spp.) that are unlikely to be contaminants. Also, a few bacterial colonies have been isolated from the interstitial water collected from the piezometers (see Materials and Methods). 16S rDNA sequences from these organisms have been obtained (data not shown), and they were identical to that of OTU 7, therefore suggesting that they could be endogenous bacteria. In conclusion, 18 and 8 sequences expected to be of endogenous origin were found in samples collected 7 and 3 m, respectively, from the gallery wall (Table 1), and they could be assigned to eight and six different OTUs, respectively. It is noteworthy that the composition of the bacterial community that we have identified is very similar to that found in a recent study of subsurface environments (51).

If these organisms are not contaminants introduced during sample processing, what is their actual origin? One hypothesis is that they became trapped 35 million years ago during the sedimentary process. A vertical migration from the surface through the overlaying Neogene sands (0 to 188 m deep) is not strictly impossible considering the time scale involved and the porosity of the sand (i.e., 200,000 years at 1 mm/year). However, starting at a depth of 188 m, the Boom clay formation is characterized by a porosity of between 10 and 20 nm, with the largest pores not exceeding 0.1 to 0.2 μm. Such a clay lattice structure makes it quite difficult for bacteria to migrate from a depth of 188 m to 224 m. Since exchanges are difficult because of the low porosity, it could then be expected that some differentiation of the communities has taken place. This could be the case, since the dominant OTU in the 7-m-deep community could not be detected in the sample collected at a depth of 3 m. On the other hand, the fact that some OTUs were retrieved in both samples is not surprising. All samples were collected at a depth of 224 m, along a horizontal core, and therefore the two bacterial communities correspond to the same sedimentary process, a few meters away. It is relatively difficult to relate the diversity estimated by PCR and cloning to the diversity of the natural community. First, a complete and thorough survey of the organisms in the clay has not been done, as only a limited number of clones have been analyzed (the redundancy of similar clones suggests, however, the presence of few different PCR sequences). Second, a linear relationship between the actual density of each species in the natural community and the amounts of its PCR products is not realistic. Divergences could be due to differences in the efficiency of lysis for different bacterial species, to the variability in the numbers of rDNA genes in different genomes (16), to differential sorbing of DNA to clay particles, and finally to differences in the yields of PCR because of differences in G+C compositions or secondary structures (unpublished results). Moreover, not all PCR products are cloned with the same efficiency. As a result, any estimation of bacterial diversity is probably only approximative, yet it is still a far better estimate than that obtained by culture methods that sample only a very small percentage (sometimes less than 0.1%) of the total viable bacteria (6). Our estimate of bacterial diversity may, however, be compared with that obtained by similar techniques but for other ecosystems. Among the 12 possible endogenous OTUs that have been sequenced, 5 were found at least twice. This is a significantly higher redundancy than that observed in more open environments, such as the ocean (12, 18) and ground surfaces (14, 37). However, this diversity compares well with that of environments such as a Yellowstone hot spring (4) and deep-sea vents (31), which also offer extreme or confined conditions. Thus, our data support the idea that the extent of diversity displayed by communities of microorganisms is related to the degree of confinement of their ecosystem.

In conclusion, our data confirm that a molecular approach involving PCR is probably superior to conventional culture methods for revealing the presence of bacteria in the environment, mostly because it is possible to identify the organisms that are present even though they are not easily culturable. However, our work also suggests that even when all the necessary precautions are taken, contamination remains a problem when dealing with samples containing very few bacteria, especially when sampling is done under difficult conditions. A precise identification of contaminants by molecular methods will remain extremely difficult as long as the sequence database does not include sequences from a significantly higher number of the organisms present in the environment.

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