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Molecular Diversity of Bacterial Communities from Subseafloor Rock Samples in a Deep-Water Production Basin in Brazil

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The deep subseafloor rock in oil reservoirs represents a unique environment in which a high oil contamination and a very low biomass can be observed. Sampling this environment has been a challenge owing to the techniques used for drilling and coring. In this study, the facilities developed by the Brazilian oil company PETROBRAS for accessing deep subsurface oil reservoirs were used to obtain rock samples at 2,822–2,828 m below the ocean floor surface from a virgin field located in the Atlantic Ocean, Rio de Janeiro. To address the bacterial diversity of these rock samples, PCR amplicons were obtained using the DNA from four core sections and universal primers for 16S rRNA and for APS reductase (*aps*) genes. Clone libraries were generated from these PCR fragments and 87 clones were sequenced. The phylogenetic analyses of the 16S rDNA clone libraries showed a wide distribution of types in the domain bacteria in the four core samples, and the majority of the clones were identified as belonging to *Betaproteobacteria*. The sulfate-reducing bacteria community could only be amplified by PCR in one sample, and all clones were identified as belonging to *Gammaproteobacteria*. For the first time, the bacterial community was assessed in such a deep subsurface environment.

Keywords: 16S rRNA gene, *aps* gene, bacterial diversity, subseafloor biosphere

Marine subsurface environments cover more than two-thirds of Earth and are considered an inhospitable habitat for most of the microorganisms. Although the subseafloor biosphere is considered the largest prokaryotic habitat, it also presents the lowest metabolic rates, indicating that most prokaryotes may be inactive or have very slow metabolism [7]. More recently, metabolically active bacterial cells have been demonstrated in sediments as deep as 400 meters below sea floor (mbsf) [32, 35]. Parkes *et al.* [25] also showed that deep sedimentary prokaryotes can have high activity, have changing diversity associated with interfaces, and are active over geological timescales. Moreover, culturable bacteria were detected in deep-sea sediment samples collected from the Nankai Trough at 4.15 mbsf with 4,791 m of overlying water [37], and also in deep biosphere of sediments from the equatorial Pacific and the Peru continental margin [8, 18]. Moreover, quantification of microbial communities using real-time PCR [33] and microbial diversity using 16S rRNA gene clone libraries [15, 25] have been explored in those marine sediments on the Peru continental margin. However, the marine deep subsurface microbial ecology still remains one of the most poorly studied environments [15, 35]. The reason for that is the difficulty of sampling the subsurface solids, which requires specialized techniques for drilling and coring. Besides that, the costs associated with these techniques often limit the number of samples that can be obtained, and to define variations in the microbiological population at different depths can be technically difficult [14].

The Brazilian oil company PETROBRAS, which is a pioneer in drilling of deep rock for offshore oil exploration at a high depth of seawater, has developed a technique to place deep wells for sampling intact cores at offshore platforms (Patent US5192167A, [6]). Studies on the microbial ecology of the reservoirs are of great interest since microorganisms have an important role as mediators of geochemical changes, including oil degradation. Serious problems faced by the petroleum industry are due to the presence of sulfate-reducing bacteria (SRB) during the different stages of oil recovery. SRB have been suspected of contributing to failure of certain enhanced (tertiary) oil recovery operations, possibly by degrading polymers used *in situ* as mobility control agents. Furthermore, sulfides produced by SRB in the reservoir can plug wells, reducing oil production, and they can also generate a deadly gas,

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hydrogen sulfide. Therefore, a prediction of the existence of these bacteria in the subseafloor rock of an oil reservoir by molecular approaches can be a warning that oil recovery can be hindered by the ubiquitous sulfate-reducing bacteria.

Previous studies have already demonstrated the presence of *Bacillus* strains in core samples from a virgin field located in the Atlantic Ocean, Rio de Janeiro, Brazil, using facilities entirely developed by the PETROBRAS Research Center for accessing deep subsurface oil reservoirs [5]. However, it is well known that classical plating techniques often underestimate the bacterial diversity, and only a small percentage of bacterial species have been cultured in the laboratory [1, 23]. Phylogenetic identification and *in situ* detection of bacterial cells without cultivation are important approaches to characterize environments where the increase in depth confers conditions excessively extreme for life, as high temperatures and pressures, and also low nutrient concentrations [24]. Therefore, the aim of this study was to characterize by molecular approaches the bacterial population of a subseafloor rock of an oil reservoir in Brazil, sampled at 2,822–2,828 m below the ocean floor surface. For this, the DNA extracting methodology has been adapted and libraries were constructed using 16S rRNA and *aps* gene sequences to assess the predominant bacterial population and the sulfate-reducing bacteria community, respectively, in this very deep subsurface environment.

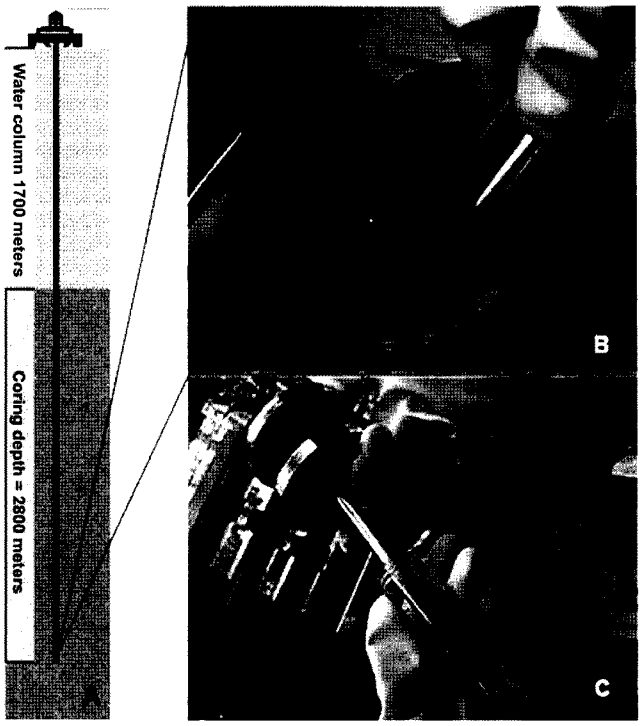

Fig. 1. A. Schematic drawing of the offshore platform. Sections were taken from the core at depth of 2,822–2,828 m below the ocean floor surface; **B**. Liner containing the core sample; **C**. Sterilized sampler used for core sampling at the laboratory.
See Materials and Methods for details.

Materials and Methods

Field Sampling

The core samples used in this study were taken from a virgin field in an offshore basin located in the Atlantic Ocean (latitude -22° 38'; longitude -39° 59'), Rio de Janeiro, Brazil, prior to any production or seawater injection, using facilities entirely developed by the PETROBRAS Research Center for accessing deep subsurface oil reservoirs. Sampling of subsurface solids requires specialized technologies for coring (collecting intact samples of subsurface materials). The coring technique used for sampling employs a hollow drill pipe and two core barrels (outer barrel and inner barrel). The outer barrel, thread together with the hollow drill pipe as the borehole is deepened. The inner barrel is stationary, covered internally with a fiber glass tube called liner, where the core samples settle in. Once the core had been brought to the surface, the liner containing the intact core was removed from the core barrel. After subsurface material had been recovered, it was subjected to processing prior to analyses or use in experiments. Processing included paring to remove outer-core material, cleaning and disinfection, and then sectioning (each section was 20 cm length and 15 cm diameter). Sterile end caps were hooked up to the core liner. Samples were transported to the laboratory, preserved in dry ice. The core sections were taken from the core at depth of 2,822–2,828 m below the ocean floor surface (Fig. 1A).

The temperature of the reservoir was 60°C, the pressure varied from 23,830 to 26,477 KPa, and the salinity was equivalent to 55 g/l of NaCl. The water column above the ocean floor was of 1,717 m. The local geology consists of sedimentary rock, which is composed mainly of massive, fine to medium grained, grayish brown to greenish gray sandstone. Cross stratified lamination was observed. At the end of the cored well, some interstratifications with marls and shale was detected followed by laminated sandstone.

In the laboratory, the outer portion of the rock samples was pared using a sterile metal spatula (Fig. 1B). The inner portions of the core were subsampled by using a sterilized sampler that consists of two steel cylinders. One cylinder is hollow (10 cm length, 14 mm outer diameter, 12 mm inner diameter), where the inner portions of the core was settled in by pushing it down against the rock sample. The second cylinder is solid (10 cm length, 11 mm diameter) and was used to pull out the inner portions inside a sterile falcon tube (Fig. 1C). In this study, four sections of a rock core were obtained from an interval between 2,822 and 2,828 m below the ocean floor surface at a Brazilian offshore oil reservoir. All samples were kept in sterile flasks and preserved at -70°C before DNA extraction.

DNA Extraction from the Rock Samples

The entire process of DNA extraction was carried out in a laminar-flow hood, and aerosol-resistant pipette tips were used to reduce the likelihood of contamination. DNA extraction was performed using 30 g of inner portions of the rock core sections taken as described above. Samples were mixed with 150 ml of the homogenization buffer based on Winogradsky's salt solution (containing, per liter, 0.25 g of K_2HPO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.125 g of NaCl, 2.5 mg of $Fe_2(SO_4) \cdot 3H_2O$, and 2.5 mg $MnSO_4 \cdot 4H_2O$), with the addition of 15 g of polyvinyl pyrrolidone (PVP) and 10 g of 2 mm glass-beads, and they were incubated for 2 h at 150 rpm at room temperature in

order to dissolve any aggregated material. The samples were then centrifuged at 4°C for 10 min at 750 ×*g* and the supernatant was kept at 4°C. Additional Winogradsky buffer (100 ml) was added to the pellet, mixed manually and centrifuged again at 4°C for 10 min at 750 ×*g*. This procedure was repeated 3 times before all supernatants were concentrated by centrifugation at 4°C for 20 min at 10,000 ×*g* in order to recover the microbial fraction. The pellet containing the microbial fraction was carefully washed in 200 ml of TE buffer [31] and resuspended in 10 ml of the same buffer. Subsequently, the cell suspension was divided in two polystyrene tubes and cell lysis was performed by adding 3 g of acid-washed glass-beads (106 microns and finer; Sigma) to each tube. These tubes were closed tightly and submitted to mechanical lysis using 3 cycles of 60 s in a MSK cell homogenizer (Braun-Melsungen) at 4,000 rpm with 10 s intervals between cycles. Samples containing the disrupted cells were kept on ice, and 180 ml of SDS 20% was added. Furthermore, they were gently mixed with 4 ml of phenol, and the glass-beads, phenol, and precipitated cell debris were separated by centrifugation at 6,000 ×*g* for 5 min. The aqueous phase was transferred to a 50-ml centrifuge tube, treated with 4 ml of chloroform:isoamylic alcohol (24:1), and centrifuged at 6,000 ×*g* for 20 min. After that, the nucleic acids were precipitated by using 0.5 ml of 5 M NaCl and 2 volumes of ice-cold ethanol for 18 h at −20°C. DNA extracts were then collected by centrifugation for 10 min at 12,800 ×*g*. The pellet was washed with 5 ml of 70% ethanol, air dried in a laminar-flow hood, and resuspended in 200 µl of TE buffer. The DNA in the bulk extract was solubilized in a water bath at 55°C for 5 min. Then, 0.1 g of CsCl was added for each 100 µl of bulk extract and kept for 1 h at room temperature. The supernatants were transferred to new eppendorf tubes and 400 µl of distilled water and 300 ml of isopropyl alcohol were added. The mixture was kept at room temperature for 30 min, harvested by centrifugation at 12,800 ×*g* for 15 min, air dried, and resuspended in 100 µl of TE.

PCR Amplification of Bacterial Community (16S rDNA)

The 16S rRNA gene sequences were amplified from the DNA extracted from the four rock samples by PCR using the universal primers and the PCR conditions described by Heuer and Smalla [13]. The 50 µl PCR reaction mix contained 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM dNTPs, 0.2 µM of each primer (U968: 5' AACGC-GAAGAACCTTAC 3' and L1401: 5' GCGTGTGTACAAGACCC 3'), 2.5 U of *Taq* DNA polymerase (Promega), and 2 µl of the DNA extract. The amplification conditions applied were as follows: denaturing step of 94°C for 2 min, followed by 35 cycles of 1 min at 94°C, annealing for 1 min and 30 s at 48°C and extension for 1 min and 30 s at 72°C, followed by a final extension at 72°C for 10 min. Negative controls (without DNA) were run in all amplifications and the presence of PCR products was checked by 1.4% agarose gel electrophoresis followed by staining with ethidium bromide.

PCR Amplification of Sulfate-reducing Bacteria Community (*apsA* Gene)

The primers F2 (5' CCAGGGCCTGTCCGCCATCAATAC 3') and R2 (5' CCGGGCCGTAACCGTCCTTGAA 3') described by Zinkevich and Beech [44], which correspond to nucleotide positions 969–992 and 1,603–1,624, respectively, of the adenosine-5'-phosphosulfate reductase alpha subunit locus (*apsA*) of *Desulfovibrio vulgaris*, were used for the amplification of a 658-bp DNA fragment. PCR amplifications were performed also as described by Zinkevich and Beech [44]. The 50 µl PCR reaction mix contained 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM dNTPs, 0.4 µM of each primer, 2.5 U of *Taq* DNA polymerase, and 2 µl of the DNA extract. The conditions applied were as follows: denaturing step of 95°C for 2 min, followed by 35 cycles of 1 min at 95°C, annealing for 1 min at 62°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 10 min. Negative controls (without DNA) were also run in all the amplifications. The PCR products were visualized by 1.4% agarose gel electrophoresis and stained with ethidium bromide.

Table 1. Rock samples used and clone libraries obtained in this study.

Rock samples	T2-1/9	T2-2/9	T2-7/9	T2-8/9	Total
	Number of clones				
Alphaproteobacteria	8	6	1	2	17
Betaproteobacteria	7	11	10	8	36
Gammaproteobacteria	1	1	2	1	5
Firmicutes	1	1	5	2	9
Actinobacteria	–	–	–	1	1
Total	17	19	18	14	68

Clone Libraries and Phylogenic Analysis

The PCR products of partial 16S rRNA (433 nt) and APS reductase (658 nt) genes from the different samples were cloned using a pGEM T-easy vector according to the instructions of the manufacturer (Promega). After the transformation into *E. coli* JM109 competent cells, all clones were selected and sequenced using an ABI Prism 3100 automatic sequencer. Sequences were compared with the GenBank database using the BLAST search. Closely related sequences were recovered from the GenBank database and aligned to our sequences using the Clustal-X software [36]. Phylogenetic trees were constructed based on partial 16S rDNA sequences, using the neighbor-joining (NJ) method, whereas MEGA 3.1 software [17] was used to calculate pairwise *p*-distance values for partial 16S rRNA gene sequences among the different species studied here. Bootstrap analyses were performed with 1,000 repetitions and only values higher than 50% were shown in the phylogenetic trees. The sequence data set was screened for potential chimeric structures by using the CHECK_ CHIMERA application at the Ribosomal Database Project Web site. Sequences found to be of potential chimeric origin were excluded from this study.

RESULTS

DNA Preparation from the Rock Samples

Four core samples obtained from the column of rock located at the interval between 2,822 and 2,828 m below the ocean floor surface were used for DNA extraction. Samples were named T2-1/9, T2-2/9, T2-7/9, and T2-8/9 (Table 1). The methodology used for DNA extraction (described in Materials and Methods) had to be adapted because of the presence in the samples of oil and low density of cells. First, the amount of rock used for DNA extraction was increased, and at least 30 g of rock was necessary to

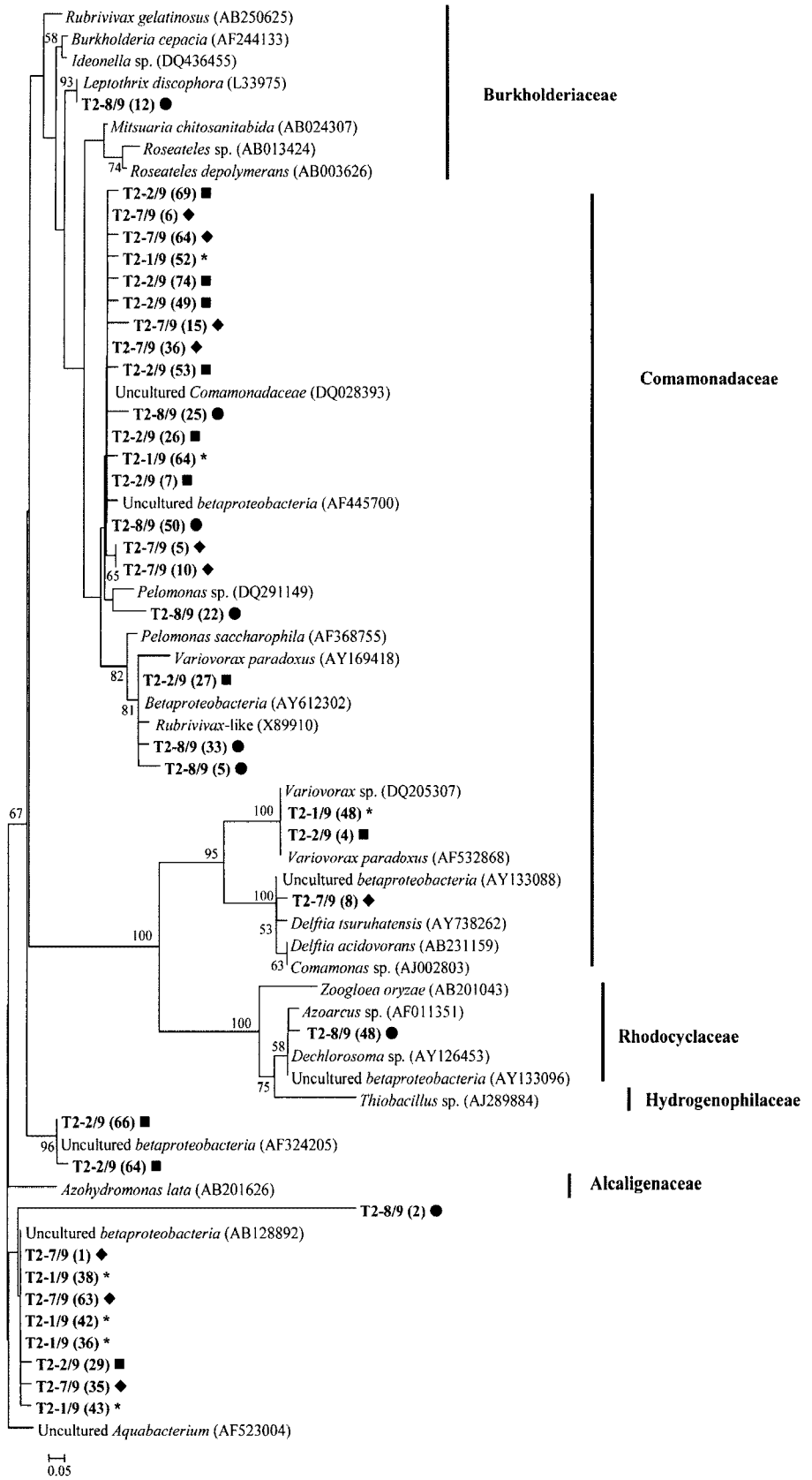

Fig. 2. Phylogenetic tree showing the affiliation of 16S rDNA clone sequences to selected reference sequences of *Betaproteobacteria*. The tree was constructed based on the neighbor-joining method. Bootstrap analyses were performed with 1,000 repetitions and only values higher than 50% are shown. 16S rDNA clone sequences are marked with different symbols based on the core samples: T2-1/9 (*); T2-2/9 (■); T2-7/9 (◆); T2-8/9 (●). Codes in parenthesis just after the core sample represent the clone numbers. The GenBank accession number of each species is enclosed in parentheses. Scale bar represents the estimated sequence divergence.

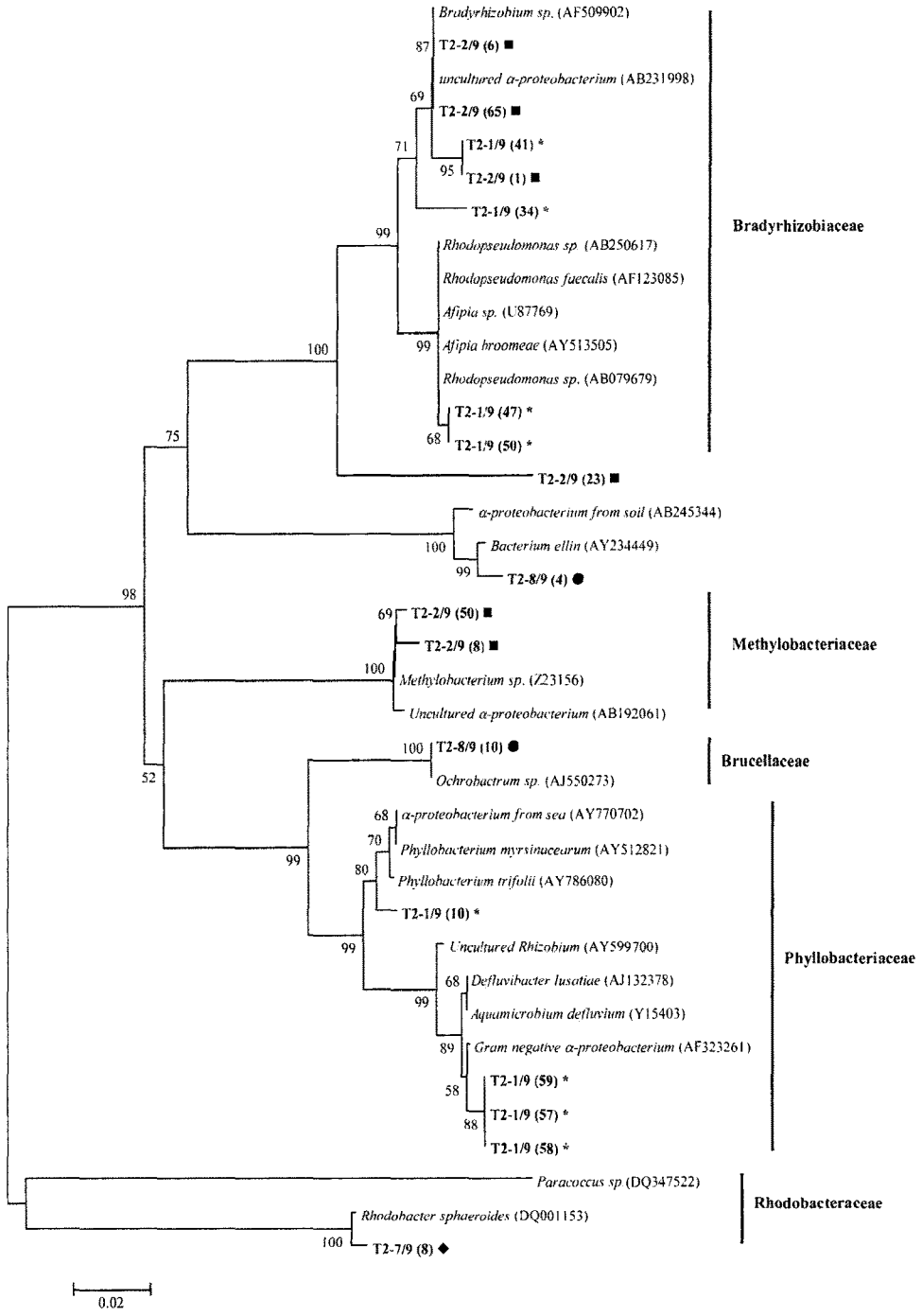

Fig. 3. Phylogenetic tree showing the affiliation of 16S rDNA clone sequences to selected reference sequences of *Alphaproteobacteria*. The tree was constructed based on the neighbor-joining method. Bootstrap analyses were performed with 1,000 repetitions and only values higher than 50% are shown. 16S rDNA clone sequences are marked with different symbols based on the core samples: T2-1/9 (*); T2-2/9 (■); T2-7/9 (◆); T2-8/9 (●). Codes in parenthesis just after the core sample represent the clone numbers. The GenBank accession number of each species is enclosed in parentheses. Scale bar represents the estimated sequence divergence.

concentrate the microbial biomass. Consequently, since not only the biomass but also the oil present in the samples was concentrated, further CsCl purification was necessary. After the DNA extraction and purification processes, DNA could not be visualized using 0.8% agarose gels, suggesting that less than 0.5 ng/μl of DNA was present in the DNA extracts. However, the amount and the quality of the DNA obtained were good enough for PCR amplifications. Positive amplifications were obtained for the four samples using universal 16S rDNA primers. On the other hand, only one sample (T2-8/9) showed positive PCR amplification for the APS reductase gene.

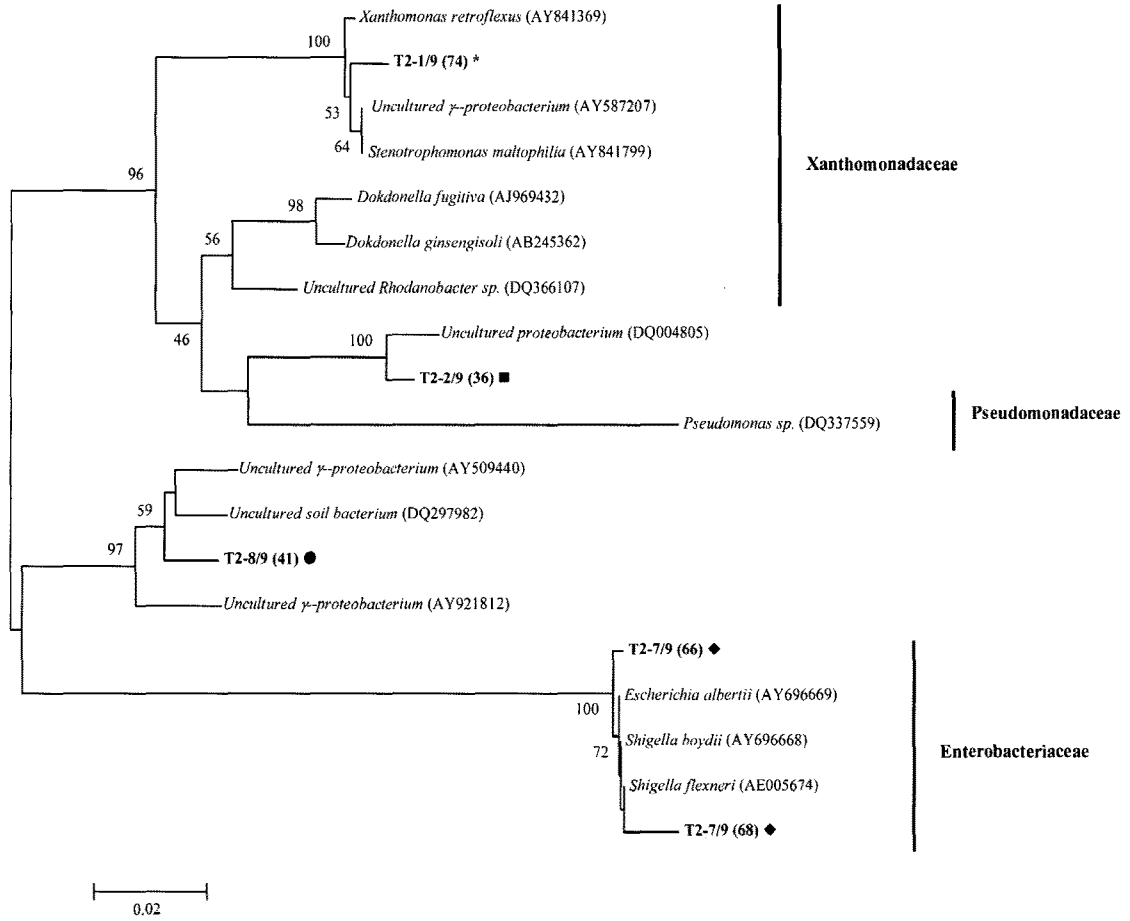

Fig. 4. Phylogenetic tree showing the affiliation of 16S rDNA clone sequences to selected reference sequences of *Gammaproteobacteria*. The tree was constructed based on the neighbor-joining method. Bootstrap analyses were performed with 1,000 repetitions and only values higher than 50% are shown. 16S rDNA clone sequences are marked with different symbols based on the core samples: T2-1/9 (*); T2-2/9 (■); T2-7/9 (◆); T2-8/9 (●). Codes in parenthesis just after the core sample represent the clone numbers. The GenBank accession number of each species is enclosed in parentheses. Scale bar represents the estimated sequence divergence.

Bacterial Diversity Based on 16S rRNA Gene Sequences

In order to characterize the bacterial diversity in the subseafloor rock samples from a deep-water production basin in Brazil, four clone libraries were constructed with the 16S rDNA PCR products obtained, representing the four core samples analyzed. Each 16S rDNA library contained between 14 and 19 clones, totalizing 68 sequences (Table 1). The phylogenetic distribution of clones based on the 16S rRNA gene sequence in the four samples was quite homogeneous, and the phylum *Proteobacteria* predominated in all samples. Most of the clones identified in this study represent *Betaproteobacteria* (36 clones, Table 1) followed by *Alphaproteobacteria* (17 clones). The *Firmicutes* were also present in all samples; however, they were more abundant in the samples T2-7/9 and T2-8/9. The number of *Alphaproteobacteria* was lower in the samples T2-7/9 and T2-8/9 than in the samples T2-1/9 and T2-2/9. The phylum *Actinobacteria* was only detected in a very low number, and only in one sample (T2-8/9, Table 1).

Phylogenetic trees were then constructed based on the most closely related sequences found in the GenBank database. When the 36 clones related to the *Betaproteobacteria* were analyzed, most of them were closely related to uncultured *Betaproteobacteria* sequences, especially related to the *Comamonadaceae* family. Affiliation to *Burkholderiaceae* was also observed among these clones. One clone clustered within the *Rhodocyclaceae* family and 11 clones could not be assigned to any known *Betaproteobacteria* family (Fig. 2).

Fig. 3 represents all clones belonging to the *Alphaproteobacteria* subdivision (17 clones, Table 1) and their closest relatives. In this group, most clones were closely related to the family *Bradyrhizobiaceae*. Four and two clones clustered within the families *Phylobacteriaceae* and *Methylobacteriaceae*, respectively. Furthermore, two clones, T2-2/9(23) and T2-8/9(4), could not be assigned to any known *Alphaproteobacteria* family (Fig. 3), and clones related to the families *Brucellaceae* and *Rhodobacteraceae* appeared only once in the libraries.

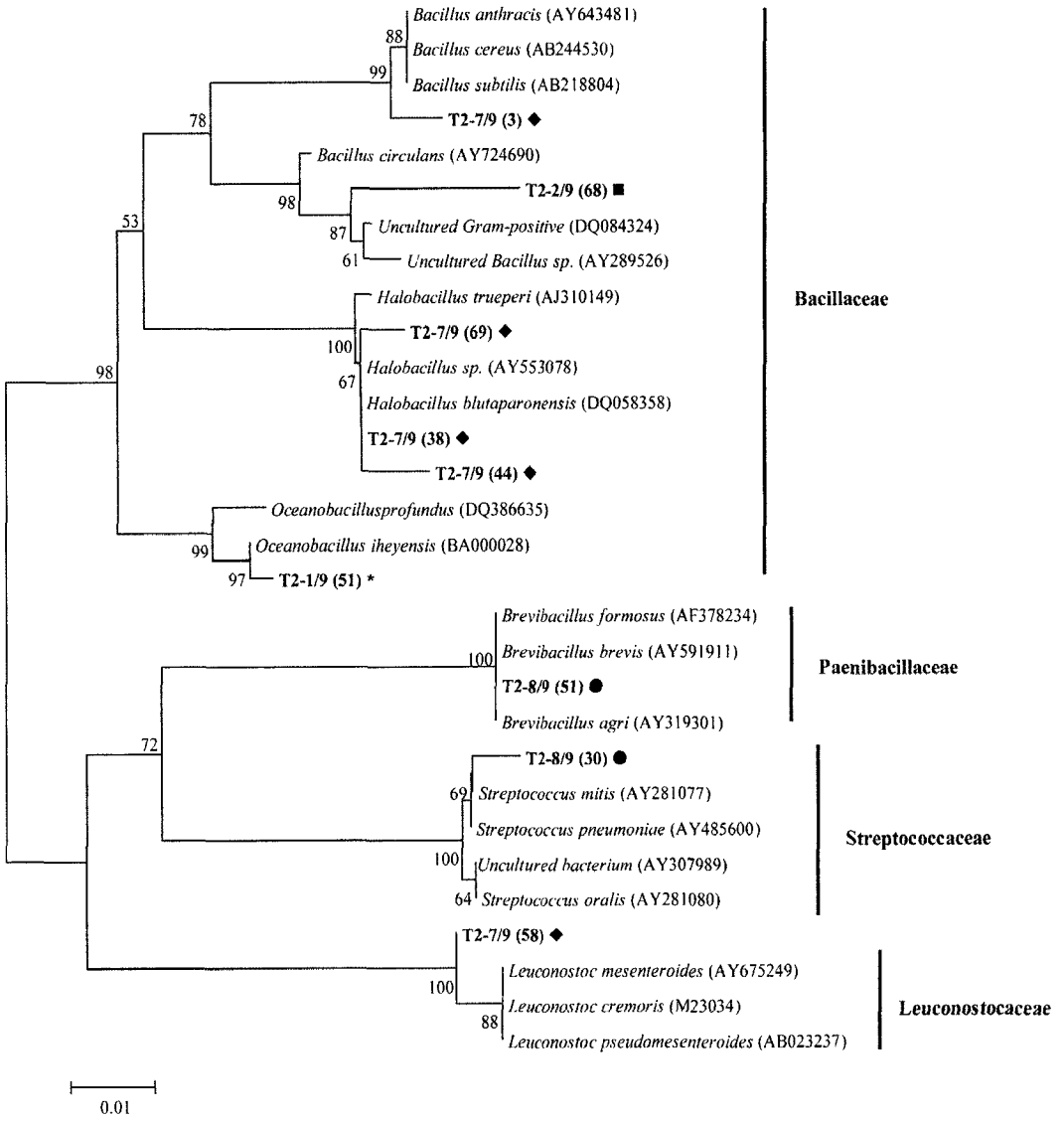

Fig. 5. Phylogenetic tree showing the affiliation of 16S rDNA clone sequences to selected reference sequences of the *Firmicutes*.
The tree was constructed based on the neighbor-joining method. Bootstrap analyses were performed with 1,000 repetitions and only values higher than 50% are shown. 16S rDNA clone sequences are marked with different symbols based on the core samples: T2-1/9 (*); T2-2/9 (■); T2-7/9 (◆); T2-8/9 (●). Codes in parenthesis just after the core sample represent the clone numbers. The GenBank accession number of each species is enclosed in parentheses. Scale bar represents the estimated sequence divergence.

The *Gammaproteobacteria* group represented only 7.3% of the total sequences obtained. However, at least one representative clone of this group was detected in each sample (Table 1). Among these clones, two sequences aligned with members to the *Enterobacteriaceae* family, one sequence was related to the *Xanthomonadaceae*, and two sequences could not be assigned to any known *Gammaproteobacteria* (Fig. 4).

Firmicutes were represented by 9 clones, six of them belonging to the *Bacillaceae* family, whereas the other three sequences were assigned to *Paenibacillaceae*, *Streptococcaceae*, and *Leuconostocaceae* (Fig. 5). *Actinobacteria* was only detected in one sample (T2-8/9) and the only sequence obtained was closely related to the high GC Gram-positive bacteria *Arthrobacter* sp. (data not shown).

Diversity of Sulfate-reducing Bacteria Based on *apsA* Gene

When the phylogenetic distribution of clones based on *aps* gene sequences was analyzed in order to detect the sulfate-reducing bacterial community in the rock samples, all the 19 sequences obtained in the T2-8/9 library were identified as belonging to the *Gammaproteobacteria* subdivision, more specifically to the genus *Desulfovibrio*. One clone was related to *D. profundus*, three clones to *D. indonesiensis*, and 15 clones to *D. desulfuricans*.

Discussion

The main purpose of environmental microbiology studies is always to identify and track specific microorganisms along with their associated physiological and metabolic activities in the various environments studied. Different methodologies based on culture-dependent or culture-independent (molecular) approaches are of great help to achieve this purpose. However, there are environmental samples where the number of microorganisms and their metabolic rates are very low, and realistic nucleic acid extraction efficiencies from soil (or sediments) border on 1 to 10% of available nucleic acid [22]. Therefore, the amount of nucleic acid available for quantitative analysis of microorganisms is many orders of magnitude below the detection threshold of different molecular assays. This was the situation found in the deep subsurface environment studied here (sample cores at 2,822–2,828 m below the ocean floor surface). Low extractable DNA yields have been also described in other deep subseafloor sites, and the microbial diversity had to be inferred from PCR-amplified DNA and the resulting clone libraries [15, 21, 25, 42]. However, the success of PCR and the construction of the libraries depend on the robust lysis of cells and the ready accessibility of targets for amplification. PCR can also be hampered when the sample has low microbial biomass content and/or inhibiting substances [38, 43], often leading to poor and irreproducible DNA extraction [3]. In this study, the extraction protocol used, including bead beating, provided low amount but sufficient DNA for PCR amplification of bacterial communities present in the different core samples. Since the detection of bacteria by PCR is a very sensitive procedure and open to contamination, carryover of PCR products from other PCR experiments in our laboratory was prevented by using sterile solutions, disposable material, and filter-containing tips throughout all experimentation. Moreover, negative control PCR experiments (without DNA extracted from the core samples) were always performed. Other concern was contamination by exogenous bacteria during sampling and, therefore, the deep subsurface sampling had been done under aseptic conditions, as detailed in Materials and Methods. Based on the results obtained in this study, the sequences are related to bacterial genera that are not viewed as likely contaminants.

Our strategy of study was supported by previous work in surface sediments and deep subsurface environments, which has also relied on 16S rDNA sequences for the analysis of the bacterial communities [4, 15, 25, 26, 30]. In general, the phylogenetic placement of subsurface sequences identified in those studies was similar to bacterial identifications inferred from physiological criteria of isolates in other subsurface systems where this phenotypic methodology was possible to be used [11]. For example, cultivations have yielded diverse bacteria (*Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*) from Leg 201 deep marine sediments [8, 35]. Moreover, Q-PCR data showed that bacteria are the dominant prokaryotes in the Leg 201 ocean-margin sediments [32]. The results of the phylogenetic analysis obtained here based on 16S rDNA sequences indicated that the bacterial diversity in the rock samples showed a wide distribution of types in the domain bacteria, with the predominance of *Betaproteobacteria*. In an expected anaerobic environment with the presence of oil, however, more members of *Gammaproteobacteria* might be expected within the clone libraries. In this study, the SRB community present in this oil reservoir could only be detected in one sample analyzed and when the *aps* primers were used. Three species belonging to the same genera were identified (*Desulfovibrio desulfuricans*, *D. profundus*, and *D. indonesiensis*). This result indicates the presence of a low number and/or diversity of this group in this environment. Parkers *et al.* [25] also observed that prokaryotes directly involved in sulfate reduction represented a small proportion of the total population found at a Peru margin site from the East Pacific Ocean.

When the diversity in bacteria was analyzed through the gene libraries obtained in this study (total of 68 sequences based on 16S rRNA), a high diversity could be observed with sequences from five major phyla. As stated before, more than half of the clones sequenced were related to the *Betaproteobacteria* subdivision, more specifically to uncultured *Betaproteobacteria* sequences found in the databases (Table 1, Fig. 2). Only one clone was affiliated with *Pelomonas* sp. (previously described as *Pseudomonas saccharophila*) and five clones with the genera *Variovorax* and *Rubrivivax* (Fig. 2). The species *V. paradoxus* has already been isolated from deep-sea sediments in the South China Sea [41], and strains previously identified as *Variovovax* sp. and the new species *R. benzoatilyticus* were described as hydrocarbon degraders [29, 34]. In addition, two other clones were closely related to *Comamonas*/*Delftia* and *Azoarcus*. Some strains identified as members of these genera have already been associated with bioremediation processes [12, 16, 20].

Concerning the *Alphaproteobacteria* subdivision, 17 clones were obtained, distributed among the four core samples, but prevailing in the libraries T2-1/9 and T2-2/9 (Table 1). Seven clones were closely related to members of the *Bradyrhizobiaceae* family (Fig. 3), two of them affiliated with members of *Rhodopseudomonas* sp., and another two with *Methylobacterium* sp. Strains belonging to both genera have already been considered as aromatic compound degraders [2, 9]. Clones T2-8/9(10) and T2-7/9(8) are closely related to sequences belonging to a diesel degrading bacterium identified as *Ochrobactrum* sp. [28] and *Rhodobacter sphaeroides* [40], respectively. Three other clones identified within

the *Alphaproteobacteria* were closely related to the *Defluvibacter/Aquamicrobium* group. *Defluvibacter lusatiae* is a Gram-negative bacterium that has been described as chlorophenol degrader [10].

Clones belonging to the *Gammaproteobacteria* and *Firmicutes* subdivisions were less abundant but also appeared in all clone libraries (Table 1). Among the five clones identified as *Gammaproteobacteria*, two of them could be related to *Xanthomonas*, *Stenotrophomonas*, and *Pseudomonas*. Hydrocarbon-degrading strains have been described as members of these genera [28].

Within the *Firmicutes* sequences found in this study, two clones could be affiliated with *Bacillus* sequences (Fig. 5). Recently, Cunha *et al.* [5] isolated and characterized different *Bacillus* strains from the rock of an oil reservoir located in a deep-water production basin in Brazil, which were able to degrade two kinds of oils and also to grow in different hydrocarbons as the sole carbon source. Two other clones were grouped within *Oceanobacillus iheyensis*; an extremely halotolerant and alkaliphilic species isolated from deep-sea sediment collected at a depth of 1,050 m on the Iheya Ridge [19], and a *Brevibacillus* sp. described as a potential asphaltene degrader [27]. Finally, only one clone could be identified as belonging to the *Actinobacteria* subdivision (Table 1). It was detected in the core sample T2-8/9 and showed high similarity to *Arthrobacter* sequences. Species from this genus had already been isolated from deep subsurface sediments [39] and showed the ability to degrade a wide range of compounds [28].

Data obtained in the present study confirm that total DNA from deep subsurface core samples obtained using deep-coring techniques can be recovered and 16S rDNA fragments can be amplified, allowing the identification of the main bacterial members present in this harsh environment. On the other hand, the SRB community present in this oil reservoir could only be detected in one sample analyzed and when the *aps* primers were used. This means that different problems caused by sulfate-reducing bacteria, such as lowering of the economic value of the produced oil, safety hazards, and plugging of the oil reservoir by insoluble iron sulfides, are not expected to happen on a large scale. However, more data are needed to determine the actual abundance of these conspicuous groups and to evaluate their roles in such a deep subsurface oil reservoir.

Acknowledgments

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