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,
hydrogen sulfide. Therefore, a prediction of the existence of these bacteria in the subsurface floor 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 subsurface floor 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 RNA and aps gene sequences to assess the predominant bacterial population and the sulfate-reducing bacteria community, respectively, in this very deep subsurface environment.

**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, is inserted into the hole. The inner barrel is stationary, covered internally with a fiber glass tube called liner, where the core samples settle into. 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.

*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.*

The core sections were taken from the core at depth of 2,822–2,828 m below the ocean floor surface. For this, 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$_2$HPO$_4$, 0.25 g of MgSO$_4$, 0.125 g of NaCl, 2.5 mg of Fe(SO$_4$)$_3$, 2.5 mg MnSO$_4$·H$_2$O), with the addition of 15 g of polyvinyl pyrrolidone (PVP) and 10 g of 2 mm glass-bead’s, and they were incubated for 2 h at 130 rpm at room temperature in