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Microbial community composition in petroleum-contaminated and uncontaminated soil from Francisco de Orellana, in the northern Ecuadorian Amazon

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Summary. The microbial compositions of two soils from the northern Ecuadorian Amazon (Francisco de Orellana province), one contaminated with petroleum and the other uncontaminated, were compared. Classical culture and molecular techniques were used to analyze microbial diversity. The cultivable *Bacteria* from contaminated soil belonged to betaproteobacteria (16.6%), gammaproteobacteria (66.6%), and Firmicutes (16,6%), whereas in uncontaminated soil, cultivable *Bacteria* were identified as gammaproteobacteria (80%) and Firmicutes (20%). Analysis of the 16S rRNA showed that in the contaminated soil proteobacterial populations (alpha-, beta- and deltaproteobacteria) were more abundant than acidobacterial populations. The Shannon index (H') was used to estimate diversity in the contaminated and uncontaminated soil. Diversity was higher in the uncontaminated ($H' = 2.16$) than in the contaminated ($H' = 1.72$) soil sample. Further studies are needed to determine whether the differences between contaminated and non-contaminated soil samples were due to spontaneous bioremediation microbial activity. [**Int Microbiol** 2008; 11(2):121-126]

Key words: 16S rRNA bacterial diversity · cultivable microorganisms · petroleum soil contamination · northern Ecuadorian Amazon

Introduction

Petroleum is a natural product resulting from the anaerobic conversion of biomass under high temperature and pressure. Although most of its components are subject to biodegradation, this occurs at relatively slow rates. Moreover, petroleum hydrocarbons are poorly degraded and have thus become the most widespread environmental contaminant [16]. These hydrocarbons can be divided into saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, porphyrins), and resins (pyridines, quinolines, carbazoles, sulfoxides, amides) [22]. These four classes differ in their susceptibility to microbial attack.

Soils are exceptionally complex, highly dynamic systems that are the product of intimate interactions between biotic and abiotic processes that have taken place over billions of years. They are highly porous materials, in which the dynamic mix of water and air-filled spaces fluctuates according to prevailing environmental conditions. The result is a spatially complex environment that drives the spatial and temporal heterogeneity of microbial diversity [18]. The immense diversity of soil-living microbes remains, to a large extent, unexplored. This is not surprising since 1 g of soil may harbor up to 10^{10} microorganisms.

Ecological diversity is considered to be a function of both the number of different types (richness or variety) of life

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forms and the relative importance of the individual elements among them (evenness or equitability). However, appropriate tools for quantifying microbial diversity in natural communities are lacking. Recently, the application of molecular techniques in environmental microbiology has provided a doorway to the discovery of organisms that are difficult to grow in the laboratory. Sequencing the microbial world will facilitate the discovery of hitherto unknown microorganisms and hitherto unknown functions of microorganisms. Microbial diversity on Earth is vast, albeit largely unexplored. To date, 55 divisions of *Bacteria* and 13 divisions of *Archaea* have been described. Soil is thought to contain more than 20 bacterial divisions, while approximately 12 division are represented in the Sargasso [6,8,23,24,26].

The ability of microbes to degrade organic contaminants into harmless constituents has been explored as a means to biologically treat contaminated environments. This approach, referred to as bioremediation, is the subject of intense laboratory research and in situ applications [25]. Bioremediation has been increasingly considered as an appropriate strategy to restore hydrocarbon-contaminated soils in ecologically protected areas from the Ecuadorian Amazon region, where both routine deliberate discharges and accidental spills are common [11]. The bioremediation procedures currently used in this region include land farming and biopiles combined with bioaugmentation (inoculation of non-indigenous microorganisms). Amazon soils seem to foster lower bacterial diversities than soils in other regions [5], suggesting that introduced bacteria are exposed to harsh conditions and competition from well-adapted native bacteria [28]. Additionally, the introduction of foreign bacterial species in ecologically protected areas, even if for bioremediation purposes, remains technically questionable.

Soils usually contain native microbiota capable of metabolizing hydrocarbons [2,8,15,28] and thus of initiating spontaneous bioremediation. In this study, culture-dependent and culture-independent approaches (DNA sequences of the 16S rRNAV3 region) were used to determine the taxonomic composition of bacterial communities present in a sample from soil contaminated with petroleum and to compare it with the bacterial communities in a non-contaminated soil sample.

Materials and methods

Soil samples. Two soil samples (5 cm deep from the surface) were collected in May 2006 in the proximity of an oil well in the northern Ecuadorian Amazon basin (Francisco de Orellana province). One sample was obtained from soil contaminated with petroleum (618.3 mg of hydrocarbon per kg) and the other sample from uncontaminated soil (70.08 mg of hydrocarbon per kg). The samples were taken from bare soils with apparently similar characteristics, and the contamination status was established by visual inspection.

Isolation of axenic bacterial cultures. A solution of 10 g of soil in 250 ml of tryptic soy broth (Difco, Detroit, MI) was prepared and incubated at 30°C for 24 h. Ten microliters of the supernatant was streaked on tryptic soy agar (Difco), and incubated at 30°C for 48 h.

DNA extraction and polymerase chain reaction. Total DNA was extracted from 0.5 g of soil samples with the UltraClean Soil DNA purification kit (Mo Bio Laboratories, Carlsbad, CA) and following the instructions provided with the kit. DNA from pure cultures was extracted with DNAzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Successful DNA extraction was determined by agarose gel electrophoresis (0.8% agarose). Polymerase chain reaction (PCR) amplification was carried out using the bacterial primers 16SV3f (5′-CCTACGGGAGGCAGCAG-3′) and 16SV3r (5′-ATTACCGCGGTGCTGG-3′). These primers amplify the V3 region corresponding to the 16S rRNA gene in the different bacterial species and to positions 341–534 in *E. coli* [17]. PCR amplification was done using pure Taq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) with 25 μM of each primer. To verify the absence of any PCR-inhibiting compounds, a control was established by mixing DNA extracted from soil with *E.coli* DNA (1:1 dilution). The PCR conditions were as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 51°C for 30 s, elongation at 72°C for 30 s, and a final extension step at 72°C for 20 min.

Cloning of amplicons. Products of three PCR reactions were pooled and ligated into plasmid pCR 2.1-TOPO (Invitrogen) and introduced into One Shot TOP10 Chemically competent *E. coli* (Invitrogen) following the instructions of the manufacturer (TOPO TA cloning kit, Invitrogen). Transformed cells were transferred to 250 μl of SOC medium and incubated at 37ºC for 1 h. A 50-μl aliquot of the transformed cells was spread on LB agar plates containing 50 μg kanamycin/ml and 40 mg XGal/ml. Recombinant colonies were isolated, purified by alkaline extraction [21], and the inserts identified by digestion with *Eco*RI and subsequent agarose gel electrophoresis (1.2% agarose). For sequencing purposes, clones were purified with the SNAP miniprep kit (Invitrogen). Inserts of 109 plasmids were sent for sequencing to Macrogen (Rockville, MD) but only 53 sequences could be obtained. The inserts were analyzed using BLAST and the neighbor-joining method. Sequences were submitted to the GenBank database and assigned accession numbers EF486799 to EF486852.

Bacterial diversity and 16S rRNA gene library comparison. Soil bacterial diversity was estimated for the two samples using the Shannon index ($H' = -\sum p_i \ln p_i$), where p_i is the proportion of clones of each phylum and the total clone number (estimated using n/N) [10]. Differences in bacterial taxonomic composition between the two libraries were assessed using the LIBSHUFF program version 0.96 [22].

Results and Discussion

The bacterial composition of contaminated and non-contaminated soil samples differed significantly (P = 0.005) as determined by the LIBSHUFF program. Phylogenetic analysis of 16SrRNA gene sequences of uncultured soil samples showed that Acidobacteria predominated in non-contaminated soil whereas in contaminated soil Proteobacteria were the most abundant (Figs. 1 and 2). Oil contamination seemed to

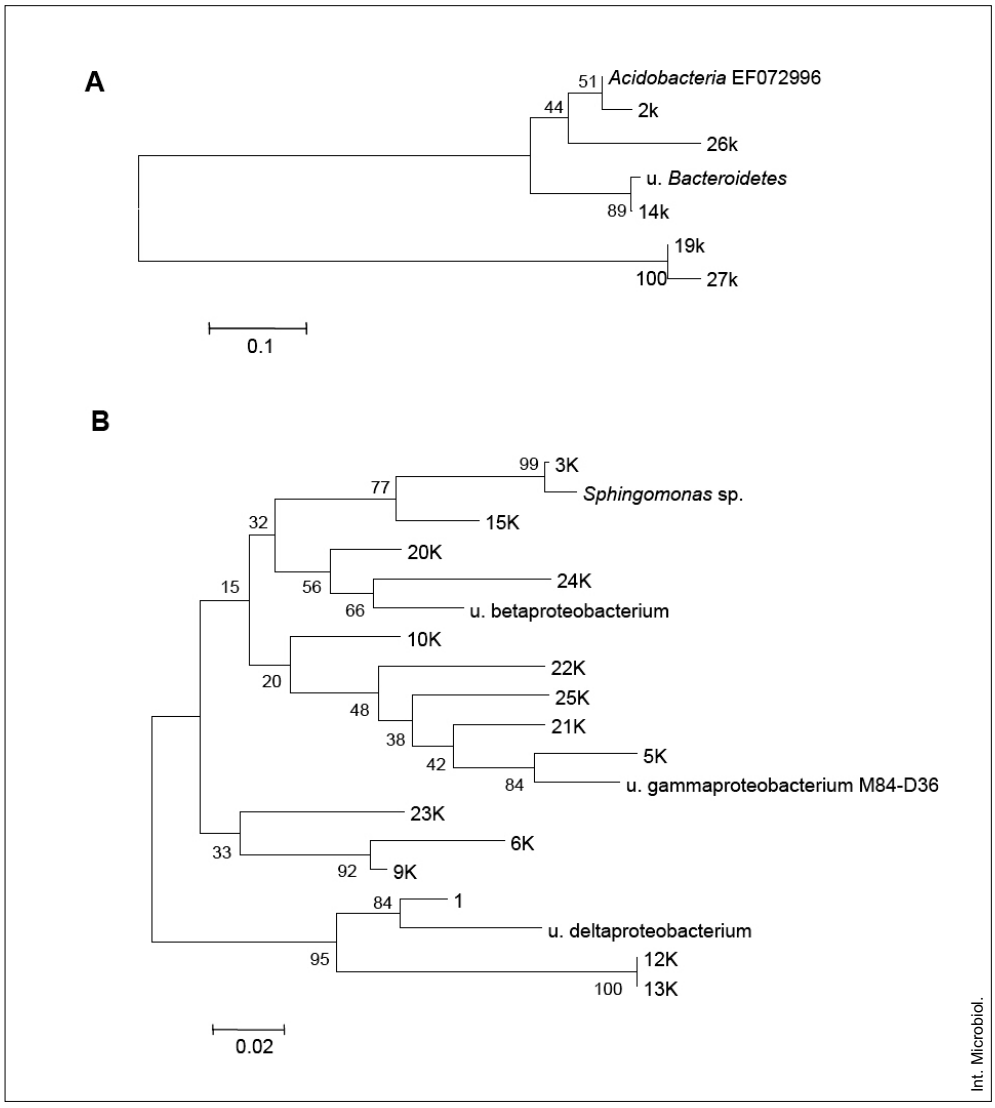

Fig. 1. Phylogenetic trees generated by the neighbor-joining method using DNA sequences corresponding to the V3 region of 16S rRNA from bacteria living in uncontaminated soil. (**A**) Acidobacteria, Bacteroidetes, and unclassified *Bacteria.* (**B**) Proteobacteria. Numbers correspond to bootstrap values.

negatively affect Chloroflexi, Cyanobacteria, Nitrospirae, and Planctomycetes, which were abundant in the non-contaminated sample but absent in the contaminated soil sample. Soil bacterial phylotype diversity, as estimated by the Shannon index [10], in the non-contaminated sample was higher ($H' = 2.16$) than that of the contaminated sample ($H' = 1.72$). These data supported the above findings, in that oil contamination seemed to reduce biodiversity and encourage the propagation of Proteobacteria, a bacterial group commonly associated with hydrocarbon degradation processes [19,28,30]. The microbiota of uncontaminated soil was unremarkable. As in soils in different parts of the world, Acidobacteria was the most abundant bacterial group [7,13,14].

Culture in nutrient media failed to reflect the diversity of the soil microbiota composition. Most *Bacteria* isolated from TSA cultures of contaminated soil were identified as gammaproteobacteria and Firmicutes, whereas culturable *Bacteria* from uncontaminated samples belonged to betaproteobacteria, gammaproteobacteria, and Firmicutes (Table 1). In agreement with previous studies, genomic analysis yielded more information than direct culture of soil samples in nutrient media [3,9,16,26]. Previous reports indicated that microbiological culture boosts fast-growing bacteria and thus may not reflect bacterial diversity in soils [4,12,29].

In some regions of the world, the proliferation of native degradative bacteria has resulted in spontaneous cleaning

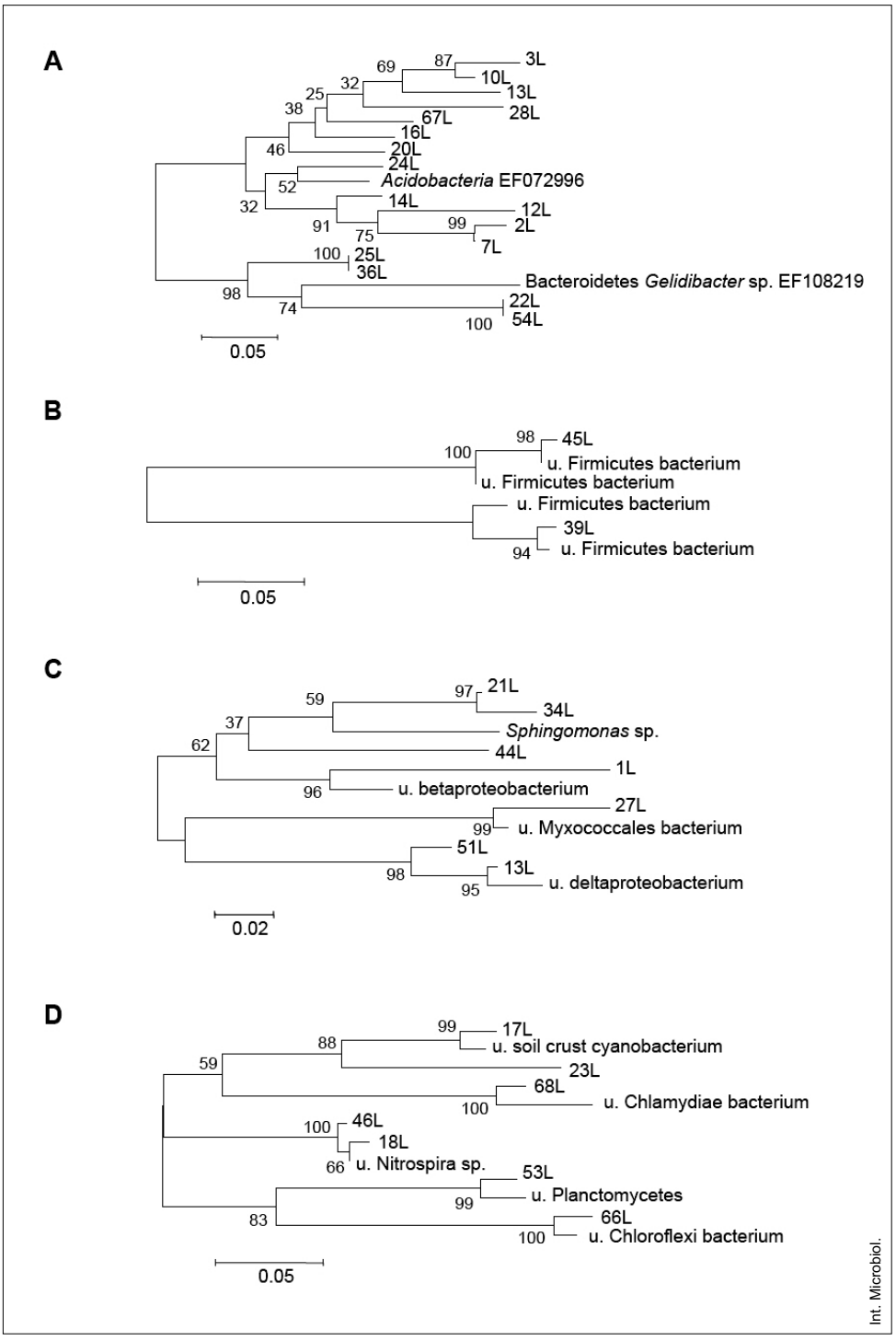

Fig. 2. Phylogenetic trees generated by the neighbor-joining method using DNA sequences corresponding to the V3 region of the 16S RNA from bacteria living in oil-contaminated soil. (**A**) Acidobacteria and Bacteroidetes, (**B**) Firmicutes, (**C**) Proteobacteria, (**D**) Cyanobacteria, Chlamydiae, Nitrospirae, Planctomycetes, and Choroflexi. Numbers correspond to bootstrap values.

events [2,20,24]. In the Amazon, this process may be enhanced by the high temperatures and humidity typical of the region [1]. Future studies should determine whether the spontaneous changes in bacterial diversity described in this report correspond to a bioremediation process. Finally, even though soil samples were collected from the same location

Table 1. Percentage of bacterial taxa detected in petroleum-contaminated and uncontaminated soil samples. Taxonomic affiliation of bacteria was determined by BLAST and by neighbor joining analysis

	Contaminated soil		Non-contaminated soil	
Groups	Cultivable isolates	16S rRNAV3 sequences	Cultivable isolates	16S rRNAV3 sequences
Alphaproteobacteria	–	23.8	–	5.6
Betaproteobacteria	16.6	9.5	–	5.6
Gammaproteobacteria	66.6	19.0	80.0	–
Deltaproteobacteria	–	14.3	–	8.3
Unclassified Proteobacteria	–	4.8	–	–
Bacteroidetes	–	4.8	–	11.1
Chloroflexi	–	–	–	2.8
Acidobacteria	–	14.3	–	33.3
Cyanobacteria	–	–	–	5.6
Nitrospirae	–	–	–	5.6
Chlamydiae	–	–	–	2.8
Planctomycetes	–	–	–	2.8
Firmicutes	16.6	–	20	5.6
Unclassified *Bacteria*	–	9.5	–	11.1

and from sites that appeared to share similar characteristics, some of the variation in microbiota composition may have been due to minor differences in soil composition.

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