CARACTERIZACION DE LA COMUNIDAD MICROBIANA RESIDENTE EN AGUAS DE PRODUCCION DE TRES CAMPOS DE EXPLOTACION PETROLERA, CON ESPECIAL ENFASIS EN GRUPOS ASOCIADOS A PROCESOS CORROSIVOS.

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CHARACTERIZATION OF MICROBIAl COMMUNITY FROM PROTUCTION WATER OF THREE OIL FIELD, EMPHASIZING IN ASOCIETED GROUPS TO CORROSION PROCCES.[*]

Silvia Juliana Salgar Chaparro & Bibiana Andrea Silva Plata[**]

Abstract

The microbial community of South American oil fields is poorly studied. This report describes the microbial composition of the heretofore unexplored Colombian oil fields, emphasizing the groups of industrial interest like sulfate reducing bacteria (SRB). PCR amplification, cloning and sequencing of 16S rDNA sequences, complemented with culture-based methods for sulfate reducing bacteria (SRB) were used for this purpose. A total of 398 clones were cloned and sequenced. A variety of sequences related to Gram positive and Gram negative bacteria were obtained. 41.6% of sequences were from Phylum *Proteobacteria* including all subclasses *α* (12.5%), *β* (9.4%), *γ* (10.4%), *δ* (8.3%) and *ε* (1%). 26% of the clones presented high identity with uncultured bacterium. A third group was formed with 18.8% of Gram positive bacteria sequences of phylum *Firmicutes*. By biochemical and molecular analysis of colonies growing on a selective medium for sulfate-reducing bacteria, 9 species were purified from 46 isolates. Analyses of results reveal some differences in species composition among Colombian oil fields, and a wide difference of bacterial composition between oil fields from North and South America. Blast searches reveal sequences commonly related to bacteria with economic importance in the hydrocarbon industry**.** This study expands the knowledge of the microbiological communities of the South American oil fields, particularly from Colombia that represents one of the most promising regions for the oil exploitation in the short time

Keywords:

Oil fields, microbial community, 16S rDNA, Microbiologically Influenced Corrosion, Sulfate Reducing Bacteria, culture-independent techniques.

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[*] Research Project

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CARACTERIZACION DE LA COMUNIDAD MICROBIANA RESIDENTE EN AGUAS DE PRODUCCION DE TRES CAMPOS DE EXPLOTACION PETROLERA, CON ESPECIAL ENFASIS EN GRUPOS ASOCIADOS A PROCESOS CORROSIVOS.[*]

Silvia Juliana Salgar Chaparro & Bibiana Andrea Silva Plata[**]

Resumen

La comunidad microbiana de los campos petroleros de Sur América están pobremente estudiados. Este informe describe la composición microbiana de tres campos petroleros de Colombia, hasta ahora inexplorados; haciendo énfasis en grupos de interés industrial, como las bacterias sulfato-reductoras (BSR). La amplificación por PCR, la clonación y secuenciación del gen 16S rRNA, complementadas con los métodos basados en cultivo de bacterias sulfato-reductoras (BSR) fueron usadas para este propósito. Un total de 398 clones fueron clonados y secuenciados. Una variedad de secuencias relacionadas a bacterias Gram positivas y Gram negativas fueron obtenidas. El 41,6% de las secuencias fueron del Filum *Proteobacteria* incluyendo todas las subclases *α* (12.5%), *β* (9.4%), *γ* (10.4%), *δ* (8.3%) and *ε* (1%). 26% de los clones presentaron una alta identidad con bacterias no cultivables. Un tercer grupo se formo con 18.8% de secuencias de bacterias Gram positivas del Filum *Firmicutes*. Para la identificación bioquímica y el análisis molecular de las colonias que crecieron en medios selectivos para bacterias sulfato-reductoras, las 46 cepas aisladas se distribuyeron en 9 especies. El análisis de los resultados evidencia algunas diferencias en la composición de las especies entre los campos petroleros de Colombia y una amplia diferencia en la composición bacteriana con los campos petroleros del Norte y Sur America. Además, se registro la presencia de secuencias comúnmente relacionadas a bacterias con importancia económica en la industria del petróleo. Estos resultados constituyen el punto de partida para el conocimiento de la composición de microorganismos de campos petroleros colombianos en el Valle del Magdalena Medio.

Palabras Clave:

Campos petroleros, comunidad microbiana, 16S DNAr, corrosion infuenciada microbiologicamente, bacterias sulfato-reductoras, técnicas independientes de cultivo.

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1. Introduction

Oil production waters may come from the formation itself (stratal), or a mixture with other sources such as ground and surface water. This aqueous solution saturated or nearly saturated with dissolved salts is commonly known as brine (Gevertz *et al*., 2000) and provides a suitable living environment for microbial life. These microorganisms may alter the conditions of the formation and the enduringness of iron structures involved in the extraction, transport, stocking and processing of the hydrocarbon-water mixture (Voordouw, 2001).

Some of the species-specific metabolic processes that cause positive or negative effects in oil industry are biotransformation, biodegradation, bioremediation, and the microbiologically influenced corrosion (MIC) (Morris *et al*., 2002; Van Hamme *et al*., 2003; Vasquez-Duhalt, 2004; Yoshida *et al*., 2005; Das and Mukherjee, 2007). Thus, the characterization of the bacterial community represents an important issue to counteract its harmful effects (Vasquez-Duhalt, 2004). Such characterization must begin by an exhaustive inventory of the microorganisms lying in production waters, by a diverse set of molecular and microbiological approaches (Burr *et al*., 2006; Nocker *et al*., 2007).

For many years, the understanding of the complexity of these communities was limited to the traditional culture-based techniques, where only 1-5% of the species were able to grow, thus underestimating the real microbial richness of these particular environments (Walker and Colwell, 1976; Ultee *et al*., 2004; Nocker *et al*., 2007).

With the development of culture-independent techniques, e.g., cloning and sequencing of marker genes, fluorescent in situ hybridization (FISH), amplified ribosomal DNA restriction analysis (ARDRA) also known as restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), it is

now possible a better understanding of the bacterial composition of uncommon and poorly explored niches (Kawai *et al*., 2002; Nocker *et al*., 2007).

16S rDNA sequence analysis has been converted in one of the most valuable tool to the molecular characterization of environmental samples including isolated strains and uncultivable microorganisms (Ward *et al*., 1990; Gillan *et al*., 1998; Singleton *et al*., 2001). Phylogenetic analyses based on 16S rDNA sequences provide a high resolving power and statistical support for either species identification or determination by comparison with already known sequences (Clarridge, 2004). GenBank, the largest databank of nucleotide sequences, has over 20 million registered sequences, 90000 of which are of the 16S rRNA gene. The Ribosomal Database Project (RDP) makes available 513,272 submissions of rRNA related data, including sequences from Eukarya, Bacteria and Archaea. This means that it is highly probable to identify unknown species by a rapid aligning of their 16S rDNA sequences against entire databases (Benson *et al*., 2003; Brito *et al*., 2006).

The Bacteria domain has shown an elevated level of diversity in all environments; however, in oil industry, there are specific groups with economic importance, particularly, the sulfate reducing bacteria (SRB). The damaging activity of this kind of microorganisms has produced important economic losses for US $4–6 billion/year, by their related corrosive processes (MIC) on field equipment and machinery (Voordouw, 2001; Zhu *et al*., 2003; Dilly *et al*., 2004; Dar *et al*., 2007).

In South America, there have been few studies evaluating the composition of the bacterial community in oil fields (Sette *et al*., 2006). The aim of this work is to describe the differences in taxa composition of Bacteria lying in petroleum production waters from three Colombian oil fields. We emphasize in the SRB population. This study improves the knowledge about the bacteria richness in Colombian oil fields, and in South America formations. Our results are based on

the cloning and sequencing of 16S rDNA obtained by conventional culture techniques and metagenomic DNA. Our genomic data was complemented with biochemical identification of cultured SRB strains.

2. Materials and methods

2.1 Sampling

14 water samples were taken from wellheads of three west Colombian oil fields. The number of samples in each oil field was 5 (F1), 2 (F2), and 7 (F3). 2 L of each sample were collected in sterile plastic bottles and transported to the laboratory under refrigeration for use before 48 h.

2.2 Media and culture conditions

1 ml of sampled water was inoculated in 9 ml of liquid Postgate medium (Postgate, 1963), under anoxigenic conditions using Nitrogen injection (Videla, 2000). Strain isolations were made by subsequent repicking in new plates with the same medium. Blackening of the medium in three successive subcultures was considered a positive growing. Electron donor and electron acceptor were tested at a concentration of 20 mM. All tasks were carried out within a Labconco anoxygenic camera and plates were stored in anaerobic jars at 32°C. SRB biochemical characterization was made as suggested Garrity (2005).

2.3 DNA extraction from water samples

Microorganisms were collected by filtering 1 L of water on a 0.22 μm-pore size membrane filter (Millipore Express PLUS Membrane). Membrane filters were washed with sterile buffered water (Clesceri *et al*., 1995), cut into small pieces and soaked in 1.8 ml of SET buffer (Sommerville *et al*., 1989). After 1-h incubation at 37°C, 360 μl of freshly prepared lysozyme solution, 150 μl of 10% SDS and 50 μl

of 20 mg/ml proteinase K were added and the suspension was incubated at 37°C for 2 h with horizontal shaking at 130 r.p.m. 400 μl of 5 M NaCl solution were added to the mixture and vortexed. 280 μl of CTAB–NaCl solution (10% CTAB in 0.7 M NaCl) were added and incubated at 65°C for 20 min. The solution was extracted by adding an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), vortexed and centrifuged at 4000g for 15 min at room temperature. The aqueous phase was extracted again with chloroform–isoamyl alcohol (24:1). A final extraction was done using an equal volume of chloroform. DNA was precipitated overnight by adding 0.6 volume of ice-cold isopropanol. After centrifugation, pellets were washed with 70% ethanol, dried and suspended in 20–200 μl of TE buffer (Rivera *et al*., 2003).

2.4 DNA extraction from BSR strains

Bacterial genomic DNA was extracted from the pure cultures using a modified method, originally described by Ausbell *et al*. (1992). One single colony was picked into 9 ml of liquid medium (Postgate, 1963) and incubated at 37°C for 6 days. Cells were concentrated by centrifugation at 12 000 x g for 2 min and pellets were resuspended in 567 μl of TE buffer. 30 μl of 10% SDS and 3 μl of 20 mg/ml proteinase K were added and the suspension was incubated at 37°C for 1 h. 100 μl of 5 M NaCl solution were added and mixed with 80 μl of CTAB–NaCl, and incubated at 65 °C for 10 min. An equal volume of chloroform-isoamyl alcohol was added and the mixture was centrifugated at 12 000 x g for 5 min. The aqueous phase was extracted with phenol–chloroform–isoamyl alcohol, as previously described. DNA was precipitated overnight by adding 0.6 volume of ice-cold isopropanol, and centrifuged. Finally, DNA was washed with 1 ml of a 70% ethanol solution and dissolved in 50 μl of sterile TE buffer (Ausubel *et al*., 1992).

2.5 PCR amplification

DNA amplification of partial 16S rRNA gene (positions 338-356 and 907-926 in E. coli 16S rDNA (Amann *et al*., 1992)) was performed by using the forward EUB 338 (5'ACTCCTACGGGAGGCAGCAG3') and reverse UNIV 907 (5'CCGTCCATTCCTTTGAGTTT3') primers. The PCR reaction mixture contained: 1X reaction buffer, 2.0 Mm MgCl2, 0.2 mM dNTPs, 0.4 µm of each primer and 1.25 U Taq DNA polymerase and 50-100 ng of template DNA. Amplification was carried out in a MJ Research PTC-100 thermocycler. After an initial denaturation step of 4 min at 94ºC, 30 PCR cycles were done, each cycle consisting of 1 min al 94ºC, 1.5 min al 61ºC and 1 min at 72ºC, ending with a final extension at 72ºC for 5 min. The resulting PCR products were resolved on a 0.8% agarose gel containing ethidium bromide. Successful PCR amplifications were purified with a Wizard® SV Gel and PCR Clean-Up System (Promega).

2.6 Cloning and Sequencing

The purified PCR products were ligated into the pGEM-T-easy vector as described (Promega), and then transformed into JM 109 competent cells. White colonies growing on LB plates containing 50 µg/ml ampicillin, IPTG and X-gal, were cultured in liquid LB for plasmid DNA isolation, following the protocol described by (Sambrook *et al*., 1989). Inserts and strains PCR products were sequenced.

2.7 Data analysis

Sequences were aligned using MUSCLE, multiple alignment software (Edgar, 2004). Initial identification of the sequences was made by performing nucleotide BLAST searches against the non-redundant database available at the NCBI website. Each sequence was checked for the presence of chimers using the CHIMERA-CHECK online analysis program of the RDP-II database (Cole *et al*., 2003). Trees were generated and constructed by performing distance matrix

analyses in PAUP 4.0 (Swofford, 2003) by using a neighbor-joining method (Saito and Nei, 1987). Relative support for the phylogram branches was estimated with 1000 bootstrap replications of the data sets with accession no.Y18095.1 (non-cultivable archaebacterium, Fig. 1) and *E. coli* (Fig. 2) as outgroups.

2.8. Nucleotide sequence accession numbers

The sequence data generated for this study were deposited in GenBank under accession numbers EU882404 to EU882508.

3. Results and discussion

3.1 Sequencing of the 16S rRNA gene of bacterial communities from production waters associated with oil wellheads

The vast majority of the microbial world cannot be characterized by traditional methods like phenotypic identification procedures, because most of microorganisms are not cultivable. In order to explore the variety of bacterial populations from oil production waters, we had recourse to 16S ribosomal DNA (rDNA)-based identification of metagenomic DNA (Weisburg *et al*., 1991; Nocker *et al*., 2007). Our study was focused in three west Colombian oil fields, where a total of 398 PCR fragments were cloned and sequenced. 250 clones were more than 90% identical to sequences already deposited in the GenBank database (Blast search), from which 96 correspond to non-redundant sequences. The minimum identity values to attribute a sequence to genus or species were 95% and 97.5%, respectively. Clones with sequence identity below 95% were assigned to the closest family (Rossello-Mora and Amann, 2001). Under this approach, of the 96 non-redundant sequences, 62 matched with published sequences at a species level and 30 sequences at genus level. Lower values than 95% identity (four clones) were assigned to the closest family. Identity values, close relatives and detection frequency of non-redundant sequences are listed in Table 1.

The distribution of the 96 non-redundant sequences, in the Bacteria domain is as follows: A major group of the sequences (41.6%) belonging to the Phylum

Proteobacteria fell into five subclasses: α (12.5%), β (9.4%), γ (10.4%), δ (8.3%) and ε (1%).

Table 1. Number of bacterial 16S rDNA sequences amplified from total of brine samples

16S rDNA-based identification[a] (Genbank accession No.)	No. of sequences[b]			Identity[c]
	F1	F2	F3	
Acetobacterium wieringae (X96955.1)			1	95
Acidovorax sp. (CP000539.1)	1			99
Agrobacterium tumefaciens (AY364329.1)	1			99
Aquabacterium hongkongensis (DQ489306.1)			1	99
Azospira oryzae (DQ863512.1)	6	1	5	97-99
Azovibrio sp. (AF011349.1)	1			96
Bacillus cereus (EU834245.1)		1		98
Bacillus circulans (AY043084.1)			1	98
Bacillus subtilis(EU532192.1)	2			99
Bacteroides sp. (AY695842.1)	4	1	3	98-99
Beta proteobacterium (EF219370.1)		1		96
Blastochloris sulfoviridis (AB110414.1)			1	95
Brachymonas petroleovorans (AY275432.1)	1			99
Brucella abortus (CP000888.1)			1	99
Brucella melitensis (AY922323.1)			1	99
Caldanaerocella colombiensis (AY464940.1)			1	97
Caloramator sp. (EU621406.1)			2	96
Clostridium hydroxybenzoicum (L11305.1)	1			97
Desulfoglaeba alkanexedens (DQ303457.1)		1		99
Desulfosalina propionicus (DQ067422.1)			1	96
Desulfotomaculum aeronauticum (AY703032.1)	3			99
Desulfotomaculum nigrificans (AY742958.1)		2		99
Desulfotomaculum geothermicum (AJ621886.1)			2	98
Desulfotomaculum sp. (DQ208688.1)	2	1	24	94-95
Desulfovibrio alaskensis (DQ867001.1)	2			99
Desulfovibrio aminophilos (AF067964.1)		2		99
Desulfovibrio desulfuricans (CP000112.1)	2			99
Desulfovibrio vulgaris (DQ067422.1)	1	3	2	99
Herbaspirillum huttiense (EU549838.1)	2		2	98-99
Moellerella wisconsensis (DQ217619.1)			1	98
Moorella glycerini (U82327.1)	1		4	90-96
Mycoplana peli (EU256383.1)			1	99
Ochrobactrum anthropi (EU622535.1)	4		9	98-99
Ochrobactrum cytisi (AM411072.1)			2	99
Ochrobactrum grignonense (AJ242581)	1			97
Ochrobactrum tritici (EU301689.1)			3	99
Petrotoga halophila (AY800102.1)	1			99
Petrotoga miotherma (L10657.1)	1			99
Petrotoga mobilis (Y15479.1)			1	98
Providencia rettgeri (EU660370.1)			5	98-99
Providencia vermicola (EF192136.1)			1	98
Pseudomonas aeruginosa (EU795302.1)	3		4	99
Pseudomonas otitidis G3 (EU301769.1)			1	99
Pseudomonas putida (EU147007.1)			2	99
Pseudoxanthomonas spadix (AM418384.1)			1	99
Roseovarius sp. (AB114421.1)		2		98
Rothia aeria (EU293888.1)			1	99
Salmonella enterica (CP000886.1)			1	99
Shigella boydii (DQ518598.1)			1	97

Silicibacter pomeroyi (CP000031.1)		1		96
Stenotrophomonas maltophilia (DQ103763.1)		2		99
Streptococcus mitis (EU200182.1)			5	98-99
Streptococcus oralis (AB355617.1)			4	99
Sulfur-oxidizing bacterium OBII5 (AF170421.1)		1		95
Syntrophobotulus glycolicus (X99706)		1		95
Syntrophorhabdus aromaticivorans (AB212873.1)	4		1	95
Thauera mechernichii (Y17590.1)			1	97
Thauera selenatis (Y17591.1)	4		2	98-99
Wolinella succinogenes (M88159.2)	3			99
Uncultured actinobacterium clone (EU522663.1)	6			98-99
Uncultured *Azospirillum* sp. (EU050699.1)			1	95
Uncultured *Chlamydiales* bacterium (AB074958.1)	1			90
Uncultured *Chloroflexi* bacterium (AM749866.1)	2		2	93-96
Uncultured Clostridia bacterium (EU050691.1)	2		6	95-99
Uncultured eubacterium (AF275925.1)	2		1	99
Uncultured *Gemella* sp. (EU071516.1)			1	95
Uncultured green sulfur bacterium (AF445702.1)		1		99
Uncultured low G+C gram-positive bacterium (AB212873.1)	1			96
Uncultured *Porphyromonadaceae* bacterium (DQ647169.1)	2			98
Uncultured *Spirochaetales* bacterium (EU266876.1)			1	99
Uncultured *Syntrophaceae* bacterium (EU043564.1)			3	91-94
Unidentified thermophilic eubacterium (AJ131537.1)	1			97
Uncultured *Thermotogales* bacterium (AM184116.1)	1	1	3	95-99
Uncultured *Thermovirga* sp. (DQ647105.1)	2			99
Uncultured bacterium clone TPD-55 16S (AY862531.1)			1	96
Uncultured bacterium clone TB3_44 (EU746739.1)	1		1	95
Uncultured bacterium clone HTB2-B12 (AB434899.1)			1	99
Uncultured bacterium clone C27 (EU234224.1)			1	98
Uncultured bacterium clone Asc-w-36 (EF632714.1)			1	98
Uncultured bacterium clone TPD-56 (AY862532.1)		2		99
Uncultured bacterium clone lg1f04 (EU037976.1)			1	95
Uncultured bacterium clone ODP1244B22.3 (AB177244.1)			10	98-99
Uncultured bacterium clone BA087 (AF323773.1)		4		97-99
Uncultured bacterium clone DQ311-48 (EU050692.1)	1		2	99
Uncultured bacterium gene clone HDBW-WB49 (AB237712.1)	2			96
Uncultured bacterium clone M40C2 (EU331373.1)	1			96
Uncultured bacterium clone SC2 (EU735602.1)			2	98
Uncultured bacterium clone DGGE band A (AB062687.1)		1		98
Uncultured bacterium clone 009H08_B_SD_P15 (CT573940.1)		1		97
Uncultured bacterium clone P2D15-723 (EF510690.1)	1			99
Uncultured hydrocarbon seep bacterium BPC028 (AF154088.1)		1		98
Uncultured bacterium clone 32b11 (EF515684.1)			1	97
Uncultured bacterium clone Zplanct55 (EF602511.1)			1	94
Uncultured bacterium clone Lupin-1130m-1-MDA (EF205276.1)		1		95
Uncultured bacterium clone LCA1-1G (EU522667.1)			1	93
Uncultured bacterium clone Fe_I21 (EU735750.1)	1			99
Total	**78**	**32**	**140**	

[a] The best matching sequence found with nBLAST
[b] Number of sequenced clones. F1, oil field 1; F2, oil field 2; F3, oil field 3.
[c] Percent identity between query and matching sequences

A second group assembled 26% of strains, attributable to uncultured bacteria. 18.8% of clones gave rise to a third group of Gram positive bacteria of the Phylum *Firmicutes*. These three groups covered more than 86% of the bacteria studied.

Besides, single sequences belonging to the Phyla *Chlamydiae, Bacteroidetes, Thermotogae, Spirochaetae, Actinobacteria, Synergitetes* and *Chloroflexi*, represented each less than 4%. 87 non-redundant sequences were considered to be representative to build a phylogenic tree, by the neighbor-joining method (Saito and Nei, 1987). As seen in Fig. 1, all the sequences retrieved in this study formed 9 genotypic clusters that are in concordance with the established phylogenetic organization of the Bacteria domain (Garrity, 2005).

Only five PCR fragments were ubiquitous and appeared frequently in the three oil fields, corresponding by Blast to *Azospira oryzae*, *Bacteroides* sp, *Desulfovibrio vulgaris*, *Desulfotomaculum* sp and uncultured *Thermotogales*. All these strains have been reported as ordinary residents in this kind of atypical environments. For example, *Azospira* sp. is known for its capability to degrade aromatic compounds like ethylbenzene and toluene (Van Hamme *et al*., 2003). *Desulfovibrio* sp and *Desulfotomaculum* sp are true dissimilatory sulfate-reducing bacteria (Postgate and Campbell, 1966).

Even if F1 and F3 shared 17 sequences (Table 1), a detailed study of the data suggests that the microbial community is quite different among fields. F3 provided 40 unshared sequences, followed by F1 (23) and F2 (16). It is noteworthy that F2 yielded a high number of exclusive sequences, despite of the fact that only two water samples were taken from that location. These unshared rDNA sequences among oil fields could be related to differences in the source and physicochemical properties of the water injection or simply to specific disparities in their geologic formation. A comparison based in the 16 rDNA sequence analysis between Colombian SRB species with those found in oil fields of western Canada (Voordouw, 1996), revealed that only *Desulfovibrio desulfuricans* is shared between the two countries.

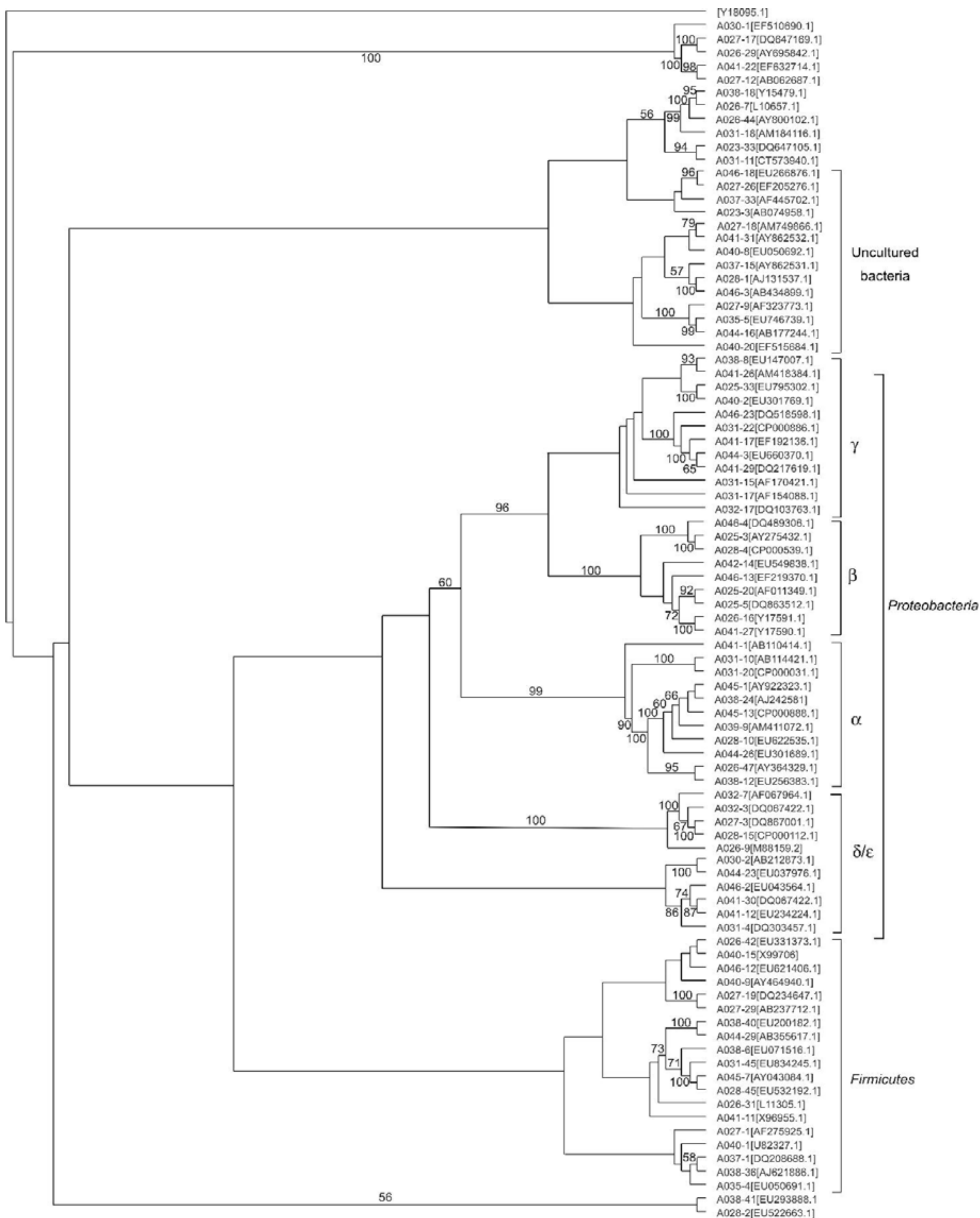

Figura 1. Neighbor-joining phylogenetic tree constructed by using bacterial 16S rDNA sequences amplified with primer pair EUB338 and UNIV907. Numbers at nodes represent the percentages of occurrence of nodes in 1,000 bootstrap trials. Only bootstrap values greater than 50% are indicated. GenBank accession numbers of sequences of the most closely related bacteria are shown in brackets. Archaeal 16S rDNA sequence served as outgroup for rooting the tree. The scale bar represents 10% estimated change.

A likely explanation is that SRB species composition is depending on the operational conditions of the production line as well as geological and environmental characteristics of each habitat.

Some bacterial strains found in this work are important for the petroleum industry. At least three species of *Clostridium* sp have been reported to increase the permeability and the emulsification of oil (Landuyt *et al*., 1995), whereas *Bacillus subtilis* has the opposite effect (Janiyani *et al*., 1994). The economical impact of these activities are critical because water-in-oil emulsions produced a various stages of exploration, production and oil recovery, represent one of the major problems of the industry (Van Hamme *et al*., 2003). Another important concern in diesel and gasoline refineries is the diminution of dissolved sulfur and nitrate (Constanti *et al*., 1996). In this study we registered many strains with high potential of desulfurization and denitrification, such as *Agrobacterium sp*, *Desulfovibrio desulfuricans*, *Pseudomonas aeruginosa*, and *Pseudomonas putida*. (Kim *et al*., 1995; Samanta and Jain, 2000). The analysis of metagenomes provides valuable information about genetic diversity, species richness, and population structure (Sekiguchi *et al*., 1998; Rondon *et al*., 2000; Nocker *et al*., 2007). Our experimental approach revealed a wide range of bacterial species living in production waters of the petroleum industry. Furthermore, it made possible to compare the community composition among individual oil fields. This knowledge is the basis for understanding the complexity of bacterial activities and interactions that allow them to exist in these extreme environments. A wide understanding of these processes will be essential for improving actions like bioremediation, the mitigation of microbiologically influenced corrosion and others issues in the entire process of petroleum exploitation (Miralles *et al*., 2004).

3.2 Isolation and characterization of a Sulfate-Reducing Bacterium (SRB)

Although the largest part of bacteria was not cultivable, we were able to recover bacterial isolates growing on a standard culture medium. Species identification was

achieved by applying physiological and morphological tests. All the strains whose cells and colonies appeared unique in size, form and color were isolated in pure cultures. 46 strains were isolated and characterized, from which 9 species of SRB were found; they belong to Phyla *Proteobacteria* and *Firmicutes* (Gram positive). Results in Table 2 summarize a number of phenotypic characteristics of the cultivable strains. Even though the biochemical analysis is not exhaustive, results listed in Table 2 are fully compatible with SRB activities. Moreover, both biochemical and molecular characterization allow concluding the correct assignment of SRB strains to *Desulfovibrio* and *Desulfotomaculum* species.

Table 2. Morphological and physiological characteristics of bacteria isolated from oil fields

Characteristics	Species								
	S1	S2	S3	S4	S5	S6	S7	S8	S9
Cell morphology	Vibrio	Vibrio	Vibrio	Vibrio	Vibrio	Vibrio	Vibrio	Vibrio	Vibrio
*Electron donors**‡									
H_2	+	+	+	+	+	+	+	+	–
Acetate	–	–	–	–	–	–	–	–	–
Ethanol	+	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+	+
Fumarate	(+)	(+)	+	–	+	+	(+)	+	nd
Succinate	+	+	+	+	+	+	+	–	+
Benzoate	–	–	–	–	(+)	–	–	–	–
*Electron acceptor**‡									
Sulfate	+	+	+	+	+	+	+	+	+
Thiosulfate	+	+	+	+	+	+	+	+	+
Fumarate	(+)	(+)	–	–	–	–	(+)	+	nd
Nitrate	–	–	+	–	–	+	–	+	–
Desulfoviridina detection†	–	–	+	+	+	+	+	+	+

‡ Determined in Postgate medium at 32 °C. –, No growth; +, good growth; (+), weak growth; nd, not determined.
† Desulfoviridin-type sulfite reductase: +, is present; –, not present.

A Blast search with partial 16S rDNA sequences from the selected group of SRB isolates was performed. The genotypic characterization of the species was based on analyses using the criterion for taxa assignment proposed by Rosello-Mora

(2001). All query sequences exhibited high identity (≥ 99%) with Genbank accessions and were correctly identified to the species level. A survey of the best matches for all 9 sequences is shown in Table 3. A striking observation is that all the cultivable strains were found in field F1, while only four belonged to F2 and 3 to F3. This marked difference in the community composition might reflect the specific conditions of each oil field for microbial life development. Known variables are physicochemical properties of each fluid (unpublished data), the carbon source, pH, dissolved ions and temperature (Huber *et al*., 2002; Crump *et al*., 2003). In addition, dissolved oxygen and requirements of bacterial consortiums to grow as a unit in these environments could be essential factors for SRB diversity in oil field waters.

Table 3Genotypic identification of oil field brine isolates

16S rDNA-based identification[a] (Genbank accession no.)	No. of sequences[b] F1	F2	F3	Identity[c]
S1-*Desulfotomaculum aeronauticum* (AY703032.1)	5	2	3	99
S2-*Desulfotomaculum nigrificans* (AY742958.1)	4	1		99
S3-*Desulfovibrio vulgaris* sub. *Vulgaris* (CP000527.1)	2	1	1	99
S4-*Desulfovibrio aminophilos* (AF067964.1)	5			99
S5-*Desulfovibrio oryzae* (AF273083.1)	6		2	100
S6-*Desulfovibrio vulgaris* sub. *Oxaminicum* (DQ122124.1)	2	1		99
S7-*Desulfovibrio alaskensis* (DQ867001.1)	2			99
S8-*Desulfovibrio desulfuricans* (CP000112.1)	5			99
S9-*Desulfovibrio termitidis* (X87409.1)	4			99
Total	**35**	**5**	**6**	

[a] The best matching sequence was found with nBLAST
[b] Number of sequenced clones. F1, oil field 1; F2, oil field 2; F3, oil field 3
[c] Percent identity between query and matching sequences

On the other hand, the same sequences were used to construct a phylogenic tree, using the neighbor-joining method (Saito and Nei, 1987). As expected, the phylogenetic analysis revealed two distinct genotypes, one consisting of *Desulfovibrio* sp and the other composed of *Desulfotomaculum* sp (Fig. 2). These two genera are economically important members of the SRB family, because they are involved in processes such metal corrosion in a variety of environments

including oil- and gas-bearing formations, soil, and domestic, industrial and mining wastewater (Chang *et al*., 2001).

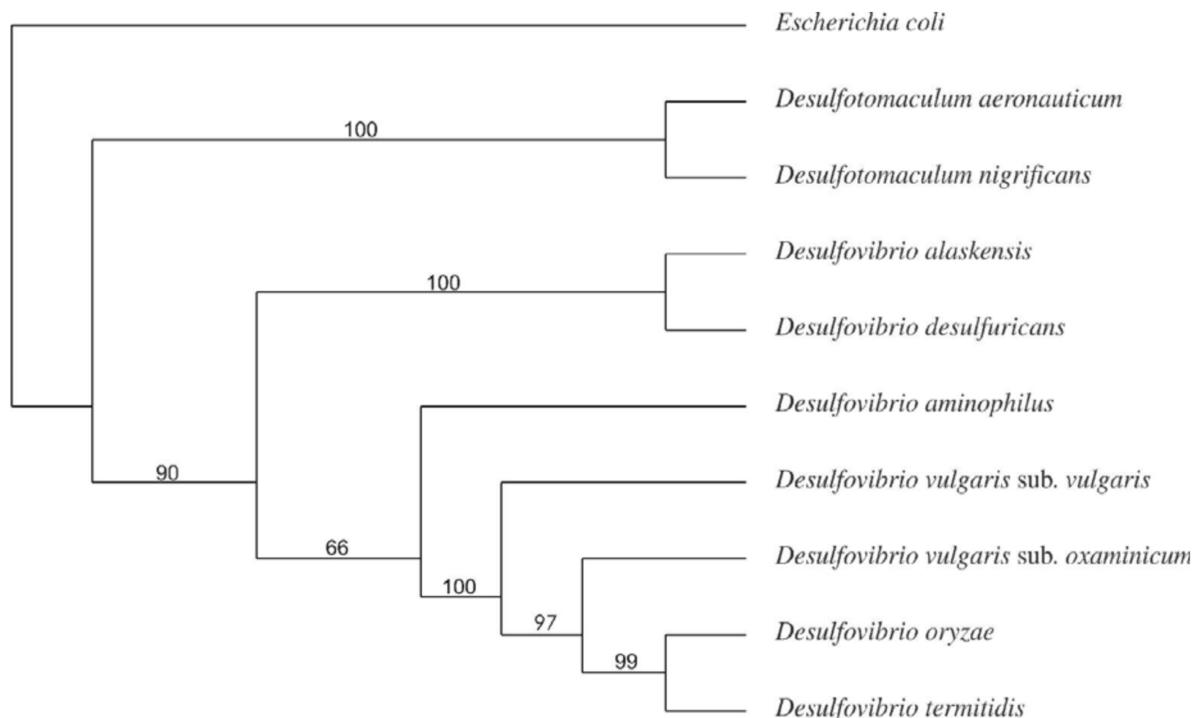

Fig 2. Neighbor-joining phylogenetic tree constructed by using bacterial 16S rDNA sequences amplified with primer pair EUB338 and UNIV907 of isolates BSR from oil field samples. Numbers at nodes represent the percentages of occurrence of nodes in 1,000 bootstrap trials. Only bootstrap values greater than 50% are indicated. GenBank accession numbers of sequences of the most closely related bacteria are shown in brackets. Nucleotides 338 to 907 of the *E. coli* sequence served as outgroup for rooting the tree. The scale bar represents 10% estimated change.

This research constitutes a striking start point to the knowledge of the microbial composition of Colombian oil fields and more widely in the Andean zone. Our study reveals an important diversity of 16S rDNA sequences, particularly related to *Proteobacteria* and *Firmicutes*. Altogether, molecular and cultured-based analyses suggest a huge complexity in community structure. Future investigations should reveal the mechanisms underlying the adaptation and the coexistence of various types of microorganisms (but not others), in specific oil fields. Further, this knowledge would contribute to develop better means of monitoring and preventing damage of structures and machinery.

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