DIVERSIDAD MOLECULAR DE LA COMUNIDAD DE ARQUEOBACTERIAS PRESENTE EN AGUAS DE PRODUCCIÓN DE TRES CAMPOS PETROLEROS COLOMBIANOS.

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Trabajo de grado para optar por el título de bióloga

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DIVERSIDAD DE ARQUEOBACTERIA BASADA EN LIBRERÍAS DEL GEN 16S rRNA EN DOS CAMPOS PETROLEROS COLOMBIANOS*

Oriana Danuta Serna Daza**

Palabras Clave:

Campos petroleros, comunidad microbiana, Archaea no cultivables, metanogénicas.

Resumen

La comunidad de arqueobacteria y su diversidad en aguas de producción en dos campos petroleros colombianos (F1 y F2) fueron estudiadas por construcción de librerías de clones 16S rRNA. Un total de 54 clones fueron identificados por búsquedas en las bases de datos rRNA. Doce filotipos del phylum Euryarchaeota fueron detectados, de los cuales 5 géneros de metanogénicas: *Methanothermobacter*, *Methanobacterium*, *Methanolinea*, *Methanococcus* y *Methanoculleus* y solamente un filotipo perteneciente al phylum Crenarchaeota. Estos resultados sugieren que la principal actividad metabólica relacionada con el dominio Archaea en los campos muestreados es la metalogénesis. Los análisis de abundancia relativa muestran que *Methanosaeta* sp, especialmente metanógenas aceticlásticas, fue el género más abundante en las librerías. Los valores del índice de Shannon (2.34 y 2.15) indicaron limitada diversidad en los dos campos, pero el campo Casabe muestra los valores de riqueza más altos (Chao1: 33). Las curvas de rarefacción e índices de cobertura para las librerías indican que las comunidades de arqueobacterias fueron bien muestreadas; sin embargo, tienen baja diversidad relativa. Un análisis de Libshuff por comparación pareada de las librerías ($P < 0.002$), indicó que las comunidades de arqueobacterias en las librerías son significativamente diferentes, con 95% de confidencia. La diversidad de las comunidades microbianas asociadas con aguas de producción de reservorios de crudo ha sido poco investigada en Sudamérica. Este estudio proporciona un conocimiento primario de la comunidad de arqueobacteria presente en aguas de producción de campos petroleros continentales en Colombia e indica una diversidad relativa distinta entre ellos.

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* Proyecto de Grado

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ARCHEAL DIVERSITY BASED ON 16S RRNA GENE LIBRARIES IN TWO CONTINENTAL COLOMBIAN OILFIELDS*****

Oriana Danuta Serna-Daza**

Keywords:

Oil fields, microbial community, uncultured Archaea, methanogens.

Abstract

The archaeal community and its diversity in production waters from two oilfields in Colombia F1 and F2) were studied by constructing archaeal 16S rRNA gene clone libraries. A total of 54 clones were identified by searches against rRNA databases. Twelve phylotypes of Phylum Euryarchaeota were detected, whereof five methanogenic genera: *Methanothermobacter*, *Methanobacterium*, *Methanolinea*, *Methanococcus* and *Methanoculleus*, and only one other phylotype belonging to phylum Crenarchaeota. These results suggest that the main metabolic activity related to the Archaea domain occurring in the sampled oilfields is methanogenesis. Analysis of relative abundances showed that *Methanosaeta* sp, especially acetoclastic methanogens, was the genus most abundant in the libraries. Values of Shannon biodiversity indices (2.34 and 2.15) indicated a limited diversity between both fields, but the Casabe oilfield had the highest community richness (Chao1: 33). Rarefaction curves and coverage indices for libraries indicated that the archaeal communities were well sampled, although with low diversity. Libshuff analyses by pairwise comparisons of libraries were calculated ($P < 0.002$), indicating that the archaeal communities in the libraries were significantly different, with 95% confidence. The diversity of microbial communities associated with production waters of petroleum crude oils has been fewer investigated in South America. This study provides a primary knowledge of archaeal community in production water from Colombian continental oilfields, and indicates a distinct relative diversity.

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1. INTRODUCTION

The diversity of microbial communities associated with production waters of petroleum crude oils has been fewer investigated in South America. A small number of reports evaluated the composition of the bacterial community (1; 2) but no one has been centered on Archaea. On the contrary, studies by different experimental approaches in most of the world's oilfields have detected anaerobic microorganisms including sulfate-reducers, fermentative bacteria, iron reducers, acetogens, and methanogens (3).

These physiological activities could have negative consequences on the economic value of a basin by biodegradation of crude oil, conversion of hydrocarbons into volatile gases, increase of oil density, sulfur content, acidity, and viscosity (4; 5). Conversely, oil recovery could be enhanced by the ability of microbial products to influence reservoir behavior and the emulsification by biosurfactants (6). Microbial methane production is a ubiquitous process in oilfields, and a variety of methanogenic microorganisms have been identified e.g., *Methanobacterium*, *Methanosarcina*, *Methanococcus*, *Methanothermobacter*, *Methanoculleus*, *Methanoplanus* and *Methanomicrobium* (3; 7).

Cultured-based methods are useful in revealing species diversity and a thorough characterization of their physiological potentials (8), well despite many microorganisms living under complex environments are nonculturable by traditional techniques. In particular, many archaeal methanogens are difficult to isolate because of their long-term growth and eventual syntopic relations of microbial consortia (9).

By molecular methods, new methanogens have been discovered and characterized (10-12). The detection and description of Archaea has been intimately coupled with the amplification by polymerase chain reaction (PCR) targeting the 16S rRNA gene used to identify, quantify, and describe populations and lineages within this domain (13). This approach made possible a reliable characterization of recalcitrant microbiota in microbial ecology studies over a wide range of environments such as soil and sediments (14), ocean (15), and hot springs (16).

Since the early twentieth century there has been prolific hydrocarbon production in the Middle Magdalena Valley of Colombia. Nevertheless, nothing is known about the richness of the microbial communities living in this petroleum basin and their taxonomical relationships with that found in other continents. The purpose of this research was to evaluate the archaeal taxonomic diversity in production waters of two Colombian oilfields, in order to contribute to the understanding of the biological processes taking place inside this environment.

2. MATERIALS AND METHODS

2.1 Sample collection

Twelve water samples were taken from wellheads of Cira Infantas (F1) and Casabe (F2) Colombian oil fields (Valle del Magdalena Medio, Departamento de Santander). The number of samples in each oil field was 5 (F1) and 7 (F2). For each sample, 2 L were collected in sterile plastic bottles, refrigerated, and transported to the laboratory to be used within 48 h.

2.2 Physicochemical characterization of production waters

By potentiometric methods, each wellhead sample was analyzed for pH, temperature, alkalinity, conductivity, sulfates, chlorides, and redox potential. The media value was achieved in each oilfield (17).

2.3 Nucleic acid extraction

Microorganisms were collected by filtering 1 L of water through a 0.22 µm-pore size membrane filter (Millipore Express® PLUS Membrane). Membrane filters were washed with sterile buffered water (18), cut into small pieces, and soaked in 1.8 ml of STE buffer (19). 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. The suspension was incubated at 37°C for 2h with horizontal shaking at 130 rpm and 400 µl of 5 M NaCl solution was added to the mixture and vortexed. Subsequently, 280 µl of 10% CTAB/0.7 M NaCl solution was 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), vortexing and centrifuging at 4000 x g 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 by adding 0.6 volume of isopropanol. After centrifugation, pellets were washed with 70% ethanol, dried, and suspended in 20–200 µl of TE buffer (20).

2.4 Construction of the 16S rRNA gene libraries

Small subunit ribosomal RNA genes of Archaea were amplified by polymerase chain reaction (PCR) using primers PARCH340f (5'-CCCTACCGGGGYGCASCAG-3') and PREA1100r, (5'-YGGGTCTCGCTCGTTRCC-3') (21). Twenty-five microliters of the reaction mixture contained 50 ng of total DNA, 1X buffer flexi, 2.0 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 μM of each primer and 1.25U of Taq DNA polymerase (Promega®). PCR amplifications were done with an initial denaturation at 94°C for 4 min, followed by 30 cycles of 1 min at 94°C, 1.5 min at 62°C and 1 min at 72°C, and a final extension at 72°C for 5 min in a MJ Research PTC-100 thermal cycler. PCR products were checked on 0.8% agarose gels stained with ethidium bromide. Amplicons (~500 ng) were purified using Wizard® SV Gel and PCR Clean-Up System (Promega®) and cloned into the p-GEM T-Easy cloning vector (Promega®), according to the manufacturer's instructions.

2.5 Sequencing and phylogenetic analysis

Sequencing of the ~760 bp inserts was performed at a contract sequencing facility (Macrogen, Seoul, Korea) with an ABI3730 XL automatic DNA sequencer. All rDNA sequences were tested for chimeras by using Bellerophon (22). Inspected sequences were used to query the GenBank database of NCBI using BLAST (23), and the Ribosomal Data Project using RDP Sequence Match to determine their phylogenetic affiliation. Partial sequences that differed by less than 3% were considered as belonging to the same phylotype. The sequences were aligned using MUSCLE version 2.1 (24), and phylogenetic and molecular analyses were conducted using MEGA version 4 (25). Phylogenetic trees were constructed using the Neighbor-Joining method; 1000 bootstrap replicates were assessed to support internal branches. Nucleotide limits similitude to define genus and species were 95% and 97.5%, respectively (26).

2.6 Statistical analyses

Phylip output format files were used to calculate distance matrices with the Dnadist version 3.5c program contained in the Phylip 3.67 program package (27). Mothur version 1.15.0 (28) was used to assign OTUs and calculate diversity indices, including abundance-based coverage Chao 1 (29) and Shannon (30) of all clone libraries. The web program Libshuff v. 0.96 (31) was used to estimate homologous and heterologous coverage of clone libraries as a function of evolutionary distance for pairwise reciprocal comparison. Differences in coverage were considered significant at P values of <0.05.

2.7 Nucleotide sequence accession numbers

The nucleotide sequences were submitted to the GenBank database under accession numbers: HQ392625-HQ392688.

3. RESULTS

3.1 Sampling site

For the study of the archaeal taxonomic diversity in production waters of Colombian oilfields, we have chosen two of the largest oilfields with reserves in the country. Both of them correspond to oilfields in a secondary recovery process in the Middle Magdalena River Valley, Colombia, located some 300 kilometers northeast of Bogotá (see Materials and Methods). In Table 1 we present the physicochemical characteristics of the sampled waters. Temperature is the foremost restrictive factor (3). As we can see in Table 1, production waters at the surface are about 36ºC for both fields, although higher values could be expected when waters have reached the total depth. F1 and F2 differ specially in alkalinity, the concentration of chlorides and sulfates and the oxidation-reduction potential. It is known that the microbial community of an oilfield depends on the physical characteristics and chemical composition of the internal environment (32). Thus, it is reasonable to expect a different composition in the microbial community between both reservoirs.

TABLE 1. Physicochemical characteristics of oilfield samples

Measurements [a]	Field [b]	
	F1	F2
Conductivity (microS/cm)	18229	21195
Alkalinity (mg/l CaCO3)	728.5	132.4
pH	7.43	7.24
Concentration Chlorides (mg/l)	6122.2	11057.4
Concentration Sulfates (mg/l)	113.71	33.1
*ORP (mV)	-297	-218
Temperature (°C)	36.5	35.7

[a] Tests
[b] Average of the results for each field
* Oxidation reduction potential

3.2 Construction and analysis of archaeal 16S rRNA gene libraries.

In order to determine the archaeal diversity between F1 and F2 oilfields, we have chosen to characterize 16S rRNA gene libraries to avoid the loss of strains by traditional culture methods. A total of 54 individual clones were sequenced of which 11 unique phylotypes were found to belong to Phylum Euryarchaeota and one to phylum Crenarchaeota. Half of the members of Euryarchaeota found in F1 and F2 fields are related to the well-known methanogen *Methanosaeta*, with sequence identities up to 97.8%. The remaining phylotypes were related to genera *Methanothermobacter*, *Methanobacterium*, *Methanolinea*, *Methanococcus,* and *Methanoculleus* (Table 2 and Fig. 1).

TABLE 2. Distribution of sequence types from the archaeal 16S rRNA gene libraries

Phylum	**Order**	**Closest species (Accession Nos.)**	F1	F2	**% Similarity**
Euryarchaeota	Methanosarcinales	Uncultured *Methanosaeta* (NR_028157)	9		90-98
		Methanosaeta thermophila (NR_028157)	3	13	97-100
		Methanosaeta concilii (NR_028242.1)	1		99
	Methanomicrobiales	*Methanoculleus receptaculi* (DQ787476)	2		98
		Methanolinea tarda (NR_028163)	2	2	97-99
	Methanobacteriales	*Methanobacterium aarhusense* (DQ649334)		1	98
		Methanobacterium alcaliphilum (AB496639)	1		99
		Uncultured *Methanobacterium* (AY607239) (DQ230925)	3	4	88-100
		Methanobacterium oryzae (NR_028171.1)	1		97
		Uncultured *Methanothermobacter* (EF100758.1) (DQ657903)(EU807735.1) (NR_028248)		6	99-100
		Methanothermobacter thermautotrophicus (EF100758)		1	97
	Methanococcales	*Methanococcus maripaludis* (AB264796)	4		99-100
Crenarchaeota		Uncultured *Thermoprotei* (HM041917.1)		1	98
		Total	**26**	**28**	

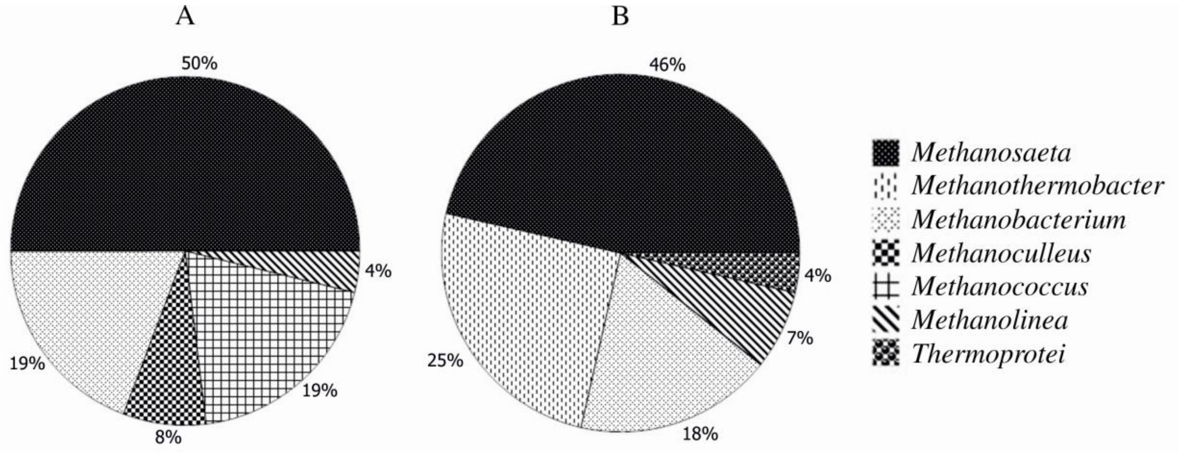

Fig.1 Phylogenetic distribution of the 16S rRNA gene sequences obtained from the clone libraries Archaea (A) field 1 and (B) field 2.

Methanosaeta, *Methanobacterium,* and *Methanolinea* were commonly detected in both oilfields, while *Methanoculleus* and *Methanococcus* were unique to F1 and *Methanothermobacter* to F2. The single phylotype of Crenarchaeota, belonging to genus *Thermoprotei,* was found in F2.

OTUs and the taxonomic diversity estimates between the two oilfields were determined with Mothur (28). 97% operational taxonomic units (OTUs) were classified into 12 OTUs for each oilfield. Calculation of Shannon index yielded virtually the same values for F1 and F2 (2.34 and 2.15, respectively). However, F1 field had the highest community richness for Chao 1 richness estimate (Table 3).

TABLE 3. Diversity indices of archaeal libraries

Library	No of clones	No of OTU	Chao1	Shannon
F1	26	12	14.5 (12.37 - 28.9)	2.34 (2.075 - 2.62)
F2	28	12	33 (17.13 - 97.95)	2.15 (1.82 - 2.5)

No of clones, number of clones sequenced
No of OTU, number of operational taxonomic units observed
Chao1, Chao1 nonparametric richness estimate and Shannon index.
Shannon, Shannon index
95% confidence interval in parentheses.

Distinct rarefaction curves for microbial communities were calculated with Mothur (Fig. 2). The number of OTUs observed versus the sampling effort show that our libraries almost reach asymptote, indicating that the archaeal community was well sampled and that the oilfields exhibit a low diversity. This is supported by diversity estimators that reveal the same tendency between fields.

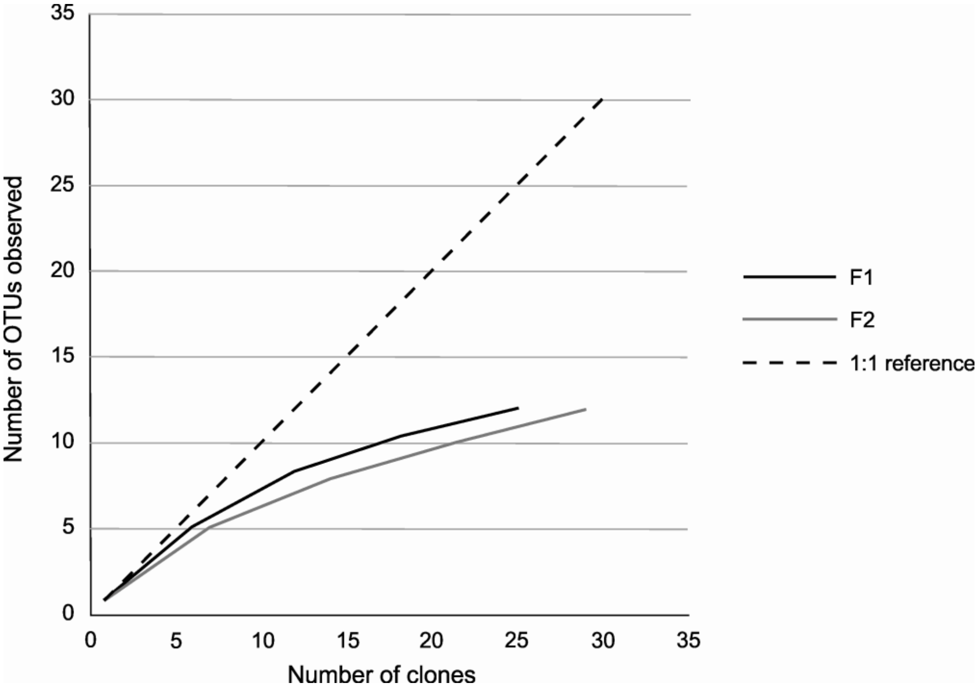

Fig.2 Rarefaction analysis comparing the relative richness of *Archaea* among clone libraries. Rarefaction curves for each clone library showing the number of clones sampled versus the number of operational taxonomic units (OTUs) within each library identified by MOTHUR program. The curve 0f 1:1 reference means that each sequenced clone belongs to unique OTU.

A pairwise comparison was achieved for the two libraries using web Libshuff (see Materials and Methods) to determine whether expected results arise from random variation or errors in sampling. The obtained P-value was found to be statistically different ($P < 0.002$), indicating that F1 and F2 were significantly different in community composition with 95% confidence.

3.3 Phylogenetic analysis of archaeal libraries

A phylogenetic tree was constructed from non-redundant sequences of F1 and F2 libraries and a set of representative sequences extracted from GenBank (Blast searches) and belonging to each of the phylotypes of Table 2. As expected, archaeal sequences were grouped in two main separate groups, Phylum Euryarchaeota and Phylum Crenarchaeota. Besides, high similarity values (>96%) were observed among the groups of Euryarchaeota found in this work according to their taxonomy (Fig. 3).

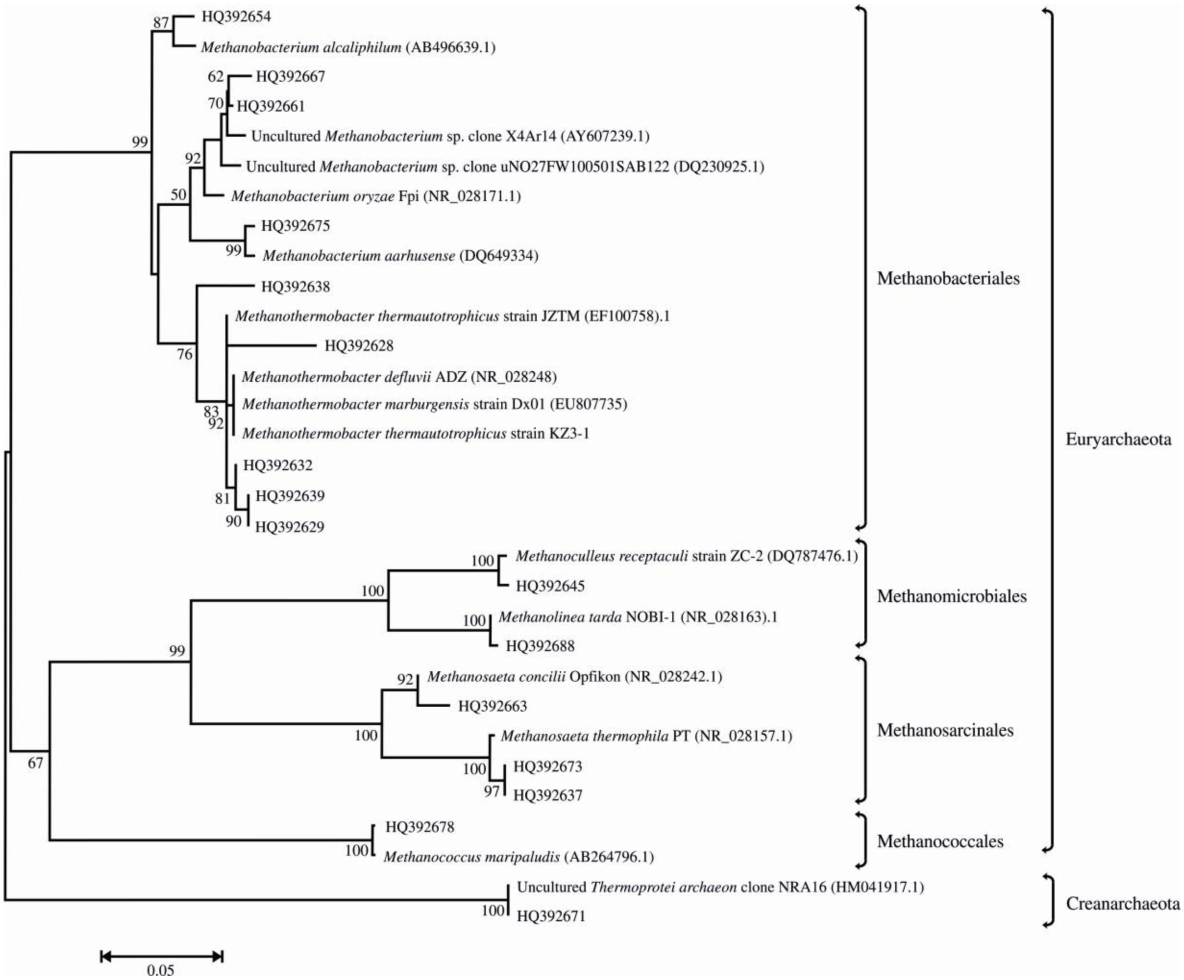

Fig.3 Phylogenetic tree showing the relationship of Archaeal 16S rRNA sequences in libraries.

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA4.0.

4. DISCUSSION

In the present work, a diversity analysis of the archaeal community from production waters was performed for two Colombian oilfields. This is the first study that describes the Archaea domain living in oilfields from South America. Our results are consistent with previous studies indicating that Euryarchaeota members are the most abundant in these environments (33-35). However, one phylotype of F2 was affiliated to *Thermoprotei* (Crenarchaeota) (Table 2).

While the connection between species abundance and metabolic activity is not necessarily direct, the physicochemical conditions of such extreme environments, e.g., the lower oxidation-reduction potential (Table 1), and the predominance of Euryarchaeota suggest that the main metabolic activity occurring in the sampled oilfields is methanogenesis concerning Archaea. It is known that crude oil biodegradation via methanogenesis occurs in subsurface petroleum reservoirs and the presence of Euryarchaeota is a good indicator of oil degradation in the sampled fields (36).

The analysis of the 16S rRNA gene libraries showed that the archaeal assemblages inhabiting these sampled oil reservoirs contained (1) mesophilic methanogens represented by *Methanosaeta*, *Methanobacterium* and *Methanolinea*, and (2) thermophilic methanogens belonging to genera *Methanothermobacter*, *Methanococcus* and *Methanoculleus*. Our findings are similar to those of other authors concerning methanogens from high temperature oilfields of Alaska and the North Sea (37) and gas fields in the North Sea (38). As previously said, temperature of production waters at the surface are about 36ºC (Table 1) but it is very probable that subsurface temperature increases to higher values, thus creating better conditions for heat resistant Archaea.

The most representative genus in the libraries was *Methanosaeta*; 19 out of 54 of the sampled clones were closely related to *M. concilii* and *M. thermophila* (Table 2), two archaeal species known to use acetate as their sole substrate for methanogenesis (39; 40). The second most abundant genus in F2 was *Methanothermobacter*, which is a common member of the microbial community associated with a high-temperature petroleum reservoir at an offshore oilfield (41), continental high temperature reservoirs (34), and gas fields (38).

The presence of methanogens in the sampled reservoirs supposes a possibility for enhancing oil recovery in these oilfields undergoing secondary recuperation. Actually, methanogenic degradation is a potentially important component of remediation, recovery, and degradation of petroleum reservoirs (42) and also a way for a subsurface biotechnology to enhance recovery of stranded energy assets (38).

Our statistical analyses reflect that the community composition differs between F1 and F2. This may be due to the differences in the physicochemical properties of each oilfield and the entire microbiota growing in each reservoir, according to their specific conditions. On the other hand, the apparent low diversity in both fields evidenced by the Shannon index may be attributed to an initial degradative process at the petroleum reservoir (36).

5. CONCLUSIONS

This study provides a primary knowledge of the archaeal diversity in northeastern Colombian oilfields, Cira-Infantas and Casabe. Similar studies in other continents report the occurrence of phylotypes related to Euryarchaeota, the most abundant phylum, specifically of methanogenic orders. Differences between the clone libraries show that each reservoir has a different community composition, possibly due as a consequence of physicochemical parameters.

Further investigation is needed to understand the physiology and ecology of those methanogenic microbial communities. The following step should be to obtain isolates in culture-dependent experiments.

6. ACKNOWLEDGMENTS

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