Taxonomic Diversity and Biodegradation Potential of Bacteria Isolated from Oil Reservoirs of an Offshore Southern Brazilian Basin

Patrícia F. Lopes-Oliveira[1*], Suzan P. Vasconcellos[1], Célio F. F. Angolini[2], Georgiana F. da Cruz[2], Anita J. Marsaioli[2], Eugênio V. Santos Neto[3] and Valéria M. Oliveira[1]

[1]Division of Microbial Resources, Research Center for Chemistry, Biology and Agriculture (CPQBA), Campinas University, Brazil
[2]Chemistry Institute, Campinas University, Brazil
[3]Petrobras R&D Center, Cidade Universitária, Brazil

Abstract

This study aimed at the taxonomic characterization of a collection with 98 bacteria isolated from oil and formation water samples from petroleum reservoirs of the Campos Basin (Brazil), as well as the evaluation of their degradation potential of petroleum biomarkers. The genomic DNA extracted from all isolates was employed in PCR reactions for amplification of the 16S rRNA gene and subsequent screening by ARDRA (Amplified Ribosomal DNA Restriction Analysis), in order to detect potentially distinct taxonomic groups. Further 16S rRNA gene sequencing and phylogenetic analysis of 39 isolates representing different ribotypes revealed that these isolates belonged to 10 different genera, encompassing Marinobacter, Halomonas, Citreicella, Stenotrophomonas, Achromobacter, Bacillus, Staphylococcus, Micrococcus, Kocuria and Streptomyces, affiliated to the three phyla Proteobacteria, Firmicutes and Actinobacteria. RAPD analysis enabled the discrimination of the isolates at the infraspecific level, allowing the identification of 45 distinct genetic band patterns. The chromatographic results showed the preference of all of bacteria to biodegrade nonadecanoic acid and squalane when grown in biomarker mixture. The results of this study provide further insight into the taxonomy of the cultivated fraction of microbial communities of Brazilian oil reservoirs and may offer potential tools for future application in bioremediation processes.

Introduction

A large part of the oil resources in our planet is constituted by heavy oil, *i.e.*, oil reservoirs that have suffered biodegradation at a certain extent [1]. There are many factors contributing to the oil degradation, including physical-chemical factors, such as the environmental pH, organic matter content, temperature, and the oil chemical composition, as well as biological factors, such as the microbial distribution in the environment, physiological and metabolic adaptations and composition of the microbial community [2].

Despite their importance for the Brazilian oil industry, the diversity of microorganisms associated with biological processes in oil reservoirs is still poorly understood. Studies related to the investigation of the biodiversity of microbial communities associated with petroleum deposits are of great significance, since they allow a deeper understanding of the structure and dynamics of those communities and the characterization of their putative functions in the environment. All this information will provide subsidies for the adoption of future measures for prevention or remediation processes of oil biodegradation and/or pipeline and associated equipment biocorrosion [3].

Recent studies from European and American research groups have demonstrated a great taxonomic and functional diversity of microorganisms in such environments, many of which representing new species and genera, including thermophylic, thermo-tolerant, aerobic and anaerobic species [4-6]. Nazina et al. [5] described a taxonomic study of a *Geobacillus* species from petroleum formation water samples collected in Russian, Kazakhstan and China. Magot et al. [4] described two new species of sulfate-reducing bacteria, *Desulfovibrio bastinii* e and *D. gracilis*, isolated from water samples collected in an oil reservoir in Congo. Other studies include a description of the species *Thermotoga subterranea* [7], *Deferribacter thermophilus* [8]

Halanaerobium congolense [9], *Petrotoga mobilis* [10] and, more recently, the species *Thermosipho geolei* [11], *Petrotoga olearia* and *P. siberica* [12], anaerobic, thermophilic and fermentative bacteria isolated from continental petroleum reservoir in Western Siberia.

More recently, investigation of the diversity of microbial communities employing cultivation-dependent and independent methods identified many aerobic genera in oil reservoirs at high temperature in the Northern Sea, including *Sphingomonas, Stenotrophomonas, Xanthomonas, Agrobacterium, Pseudomonas, Bacillus, Microbacterium, Marinobacter*, among others [13].

In Brazil, studies of microbial communities associated with oil reservoirs, using either conventional culturing techniques or cultivation-independent methods, are scarce. Recent studies developed by our research group revealed the recovery and/or detection of a wide variety of bacteria in oil samples from deep wells with different degrees of biodegradation (non-biodegraded and highly biodegraded) from the Campos Basin [14,15]. These microorganisms were shown to be related to the bacterial genera *Acidithiobacillus, Acinetobacter, Arcobacter, Marinobacter, Alicyclobacillus, Bacillus, Halanaerobium, Leuconostoc, Streptococcus, Propionibacterium, Rhodococcus* and *Streptomyces*,

distributed in 3 different phyla. Additionally, some of these bacteria were shown to be exclusive to the biodegraded oil sample, such as *Acinetobacter*, *Bacillus* and *Streptococcus* [15]. Species of *Acinetobacter* and *Bacillus* have been associated with oil and hydrocarbon degradation in many other studies [16-19]. Further biodegradation studies, performed with bacterial isolates obtained from the same petroleum samples described in Sette et al. [15], showed the recovery of bacteria capable of degrading different petroleum biomarkers [20,21]. Another recent study, using samples from a deep subsurface reservoir rock (2,800 m below seafloor) in Campos Basin [22], revealed the presence of bacteria related to the genera *Variovorax* and *Rubrivivax*, described as hydrocarbon degraders [23,24], and to the genera *Comamonas* and *Azoarcus*, associated to bioremediation processes [25,26]. Members of the Alphaproteobacteria class belonging to the genera *Bradyrhizobium*, *Rhodopseudomonas*, *Phyllobacterium* and *Methylobacterium*, known as aromatic compound degraders, and *Defluvibacter,* described as chlorophenol degraders, were also detected by Von der Weid et al. [22]. In addition, several different *Bacillus* strains isolated from deep reservoir rocks showed the ability to degrade oil and grow in different sources of hydrocarbons [27].

In this context, the present study aimed to characterize the taxonomic and genetic diversity of aerobic bacteria isolated from oil reservoirs of the Campos Basin and to assess their potential for degradation of petroleum biomarkers.

Material and Methods

Sampling and bacterial isolation

Oil and formation water samples were obtained in July of 2005 from five production reservoirs in an oil field from the Campos Basin (Macaé, RJ, Brazil), with logistic support of CENPES/Petrobras. Reservoirs from these wells differ in temperature and depth, allowing variable levels of oil degradation. Special operations made before sampling assured the origin of the oil from a specific production interval. Strict procedures were followed during sampling in order to avoid contaminations. Samples were collected in triplicate using 500 ml sterilized Schott bottles, which were completely filled with the samples in order to prevent oxygen influx. The samples were kept on ice during transportation to the laboratory, and stored at room temperature for further enrichment and isolation assays. A detailed coverage on the study area, geological and geochemical background, as well as the characteristics of the petroleum reservoirs and geochemical properties of oil samples were given in Vasconcellos et al. [21].

A collection of 98 bacterial isolates was analyzed in this work. From these, 71 strains were isolated from aerobic enrichments using the oil and formation water samples as inoculum, according to protocols described by Vasconcellos et al. [21]. Aliquots (100 μl) of the microbial enrichment were plated on nutrient agar (Difco), trypticase soy agar (Difco), marine agar (Difco), BHI (Difco) and GYM (glucose 4 g, yeast extract 4 g, malt extract 10 g, calcium carbonate 2 g, distilled water to 1000 ml, pH 7.2). The media NA and GYM were used with and without 1.5% NaCl supplementation. The plates were incubated at 28°C, 35°C and 50°C, in duplicate, for 10 days. Microbial growth was monitored every 2 days. Isolates obtained were further streaked onto the surface of fresh plates and checked for purity prior to subsequent molecular identification. The remaining 27 strains were isolated previously from the same oil and formation water samples [28]. The bacterial strains were preserved at -80°C in the isolation medium added by 10% glyceroland deposited at the Brazilian Collection of Environmental

and Industrial Microorganisms (CBMAI/UNICAMP, Brazil), under the acronyms listed in table 1.

Bacterial DNA extraction and PCR amplification

After bacterial growth on agar plates, genomic DNA of pure culture was isolated using one of the protocols previously described by Pitcher et al. [29] Young and Blakesley [30] and Pospiech and Neumann [31]. PCR amplification of 16S rDNA gene fragments was performed using the primers 27F [32] and 1401R [33], homologous to conserved regions of the 16S rRNA gene of the Bacteria Domain. Fifty μl reaction mixtures containing 50-100 ng of genomic DNA, 2 U of Taq DNA polymerase (Invitrogen), 1X Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mix (GE Healthcare) and 0.4 μM each primer. The amplification program consisted of 1 cycle at 95°C for 2 min, 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 3 min and 1 cycle of final extension at 72°C for 3 min, in an Eppendorf thermal cycler. PCR amplification of 16S rRNA gene fragments was confirmed on 1% agarose gel stained with ethidium bromide (0.1 mg/ml).

Primers gyrB UP-1 and UP-2r [34] were used for the amplification of DNA gyrase subunit B genes of the isolates identified as belonging to the *B. pumilus*/*B. safensis* group based on 16S rDNA sequences. Twenty five-μl reaction mixtures contained 50 ng of genomic DNA, 2 U of *Taq* DNA polymerase (Invitrogen), 1X *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mix (GE Healthcare) and 0.4 μM each primer. The PCR amplification program consisted of 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 2 min and 1 cycle of final extension at 72°C for 7 min, in an Eppendorf thermal cycler. PCR amplification of *gyr*B gene fragments was confirmed on 1% agarose gel stained with Sybr Safe (Invitrogen).

Primer sets BT1/BT2r and BC1/BC2r [35], for *Bacillus thuringiensis*and *Bacillus cereus,* respectively, were used for PCR amplification of the gyrase genes. Fifty-μl reaction mixtures contained 50 ng of genomic DNA, 2.5 U of *Taq* DNA polymerase (Invitrogen), 1X *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mix (GE Healthcare) and 0.4 μM each primer. The PCR program consisted of 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 55°C for 1.5 min, 72°C for 2.5 min and 1 cycle of final extension at 72°C for 7 min, in an Eppendorf thermal cycler. PCR amplification of *gyr*B gene fragments was confirmed on 1% agarose gel stained with Sybr Safe (Invitrogen).

ARDRA

ARDRA (Amplified Ribosomal DNA Restriction Analysis) was employed in order to select isolates representing potentially different taxonomic groups for subsequent sequencing and identification. The PCR products were digested separately using three restriction enzymes, *Msp* I, *Hae* III and *Alu* I (GE Healthcare), at 37°C for 2 h. Ten μl reaction mixtures contained 5 μl (~0.5 to 1.0 μg) PCR products, 3 U restriction enzyme and 1 μl enzyme buffer (10X). Digestion products were run in electrophoresis at 120 V for 2 h 40 min in 2.5% agarose gel stained with ethidium bromide. Polymorphic profiles were then visualized on a UV transilluminator and documented using the equipment BioImaging Systems UVP (UVP, Upland, CA, USA).

Sequencing and phylogenetic analysis

16S rDNA fragments of isolates exhibiting distinct ARDRA patterns (ribotypes) were further purified using mini-columns (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) and subjected to sequencing in an automated sequencer (MegaBase 500, GE Healthcare). The sequencing reactions were performed with

Deposit number	Identification	16S rRNA / GyrB accession number	ARDRA profile	[b]Origin of strains	Culture medium/ growth temperature
CBMAI 960	*Bacillus safensis* SG 1	GQ272660 JQ183055	8	P4	NA, 28ºC
CBMAI 961	*Bacillus safensis* SG 2	GQ272661	8	P5	NA, 28ºC
CBMAI 962	*Bacillus safensis* SG 3	GQ272662	8	P3	NA, 28ºC
CBMAI 963	*Bacillus safensis* SG 4	GQ272663	8	P2	NA, 28ºC
CBMAI1278	*Bacillus safensis* SG 5	JQ183021 JQ183057	7	P2	NA, 28ºC
CBMAI 964	*Bacillus safensis* SG 6	GQ272664	5	P2	NA, 28ºC
CBMAI1279	*Bacillus safensis* SG 7	[a]	8	AF	NA, 28ºC
CBMAI1280	*Bacillus safensis* SG 8	[a]	8	AF	NA, 28ºC
CBMAI1281	*Bacillus safensis* SG 9	[a]	8	AF	NA, 28ºC
CBMAI 965	*Bacillus safensis* SG 10	GQ272665 JQ183053	5	AF	NA, 28ºC
CBMAI1282	*Bacillus safensis* SG 11	[a]	8	AF	NA, 28ºC
CBMAI 966	*Bacillus safensis* SG12_1	GQ272666	8	AF	NA, 28ºC
CBMAI1283	*Bacillus safensis* SG12_2	JQ183020 JQ183052	5	AF	NA, 28ºC
CBMAI 967	*Bacillus safensis* SG 13	GQ272667	5	AF	NA, 28ºC
CBMAI1284	*Bacillus safensis* SG 14	[a]	8	AF	NA, 28ºC
CBMAI1285	*Bacillus thuringiensis* SG 15	[a]	12	P3	NA, 28ºC
CBMAI 968	*Bacillus safensis* SG 16	GQ272668	8	AF	NA, 28ºC
CBMAI1286	*Bacillus safensis* SG 18	[a]	5	AF	NA, 28ºC
CBMAI1287	*Bacillus thuringiensis*SG 20	[a]	12	P2	NA, 28ºC
CBMAI1288	*Bacillus thuringiensis*SG 21	JQ183025	12	P2	NA, 28ºC
CBMAI1323	*Bacillus simplex*SG 23_2	JQ183028	15	P3	TSA, 28ºC
CBMAI1324	*Bacillus sphaericus*SG 24	[a]	14	P1	NA, 28ºC
CBMAI1325	*Bacillus sphaericus*SG 25	JQ183027	14	P1	NA, 28ºC
CBMAI 969	*Bacillus safensis* SG 26	GQ272669	9	P1	NA, 28ºC
CBMAI 970	*Bacillus safensis* SG 27	GQ272670	8	P1	NA, 28ºC
CBMAI1289	*Bacillus safensis* SG28	[a]	8	AF	NA, 28ºC
CBMAI 971	*Bacillus safensis* SG 29	GQ272671	9	AF	NA, 28ºC
CBMAI 972	*Bacillus safensis* SG 30	GQ272672 JQ183056	6	AF	BHI, 28ºC
CBMAI1290	*Bacillus safensis* SG 31	[a]	9	AF	NA, 28ºC
CBMAI 973	*Bacillus safensis* SG 32	GQ272673	9	AF	NA, 28ºC
CBMAI1291	*Bacillus safensis* SG 33	[a]	8	AF	NA, 28ºC
CBMAI 974	*Bacillus safensis* SG 34	GQ272674	9	AF	NA, 28ºC
CBMAI 975	*Bacillus safensis* SG 35	GQ272675	9	AF	NA, 28ºC
CBMAI1292	*Bacillus safensis* SG 36	[a]	9	AF	NA, 28ºC
CBMAI1321	*Stenotrophomonas maltophilia*SG 40	JQ183019	3	P1	NA, 28ºC
CBMAI1293	*Bacillus safensis* SG 41_1	[a]	5	AF	NA, 28ºC
CBMAI1294	*Bacillus safensis* SG 41_2	[a]	5	AF	TSA, 28ºC
CBMAI 976	*Bacillus safensis* SG 42_1	GQ272676	9	AF	NA, 28ºC
CBMAI 977	*Bacillus safensis* SG 43_1	GQ272677	9	AF	NA, 28ºC
CBMAI1318	*Bacillus safensis* SG 43_2	[a]	6	AF	TSA, 28ºC
CBMAI1295	*Bacillus safensis* SG 46	[a]	9	AF	NA, 28ºC
CBMAI1326	*Achromobacterxylosoxidans* SG47_1	JQ18301 7	2	P1	NA, 28ºC
CBMAI1319	*Bacillus* sp. SG 47_2	JQ183022 JQ183051	10	P1	NA, 28ºC
CBMAI 978	*Bacillus safensis* SG 49	GQ272678	9	P3	NA, 28ºC
CBMAI 979	*Bacillus safensis* SG 50	[a]	9	P5	NA, 28ºC
CBMAI 980	*Bacillus safensis* SG 51_1	GQ272680	9	P5	NA, 28ºC
CBMAI1296	*Bacillus safensis* SG 51_2[a]	[a]	7	P5	NA, 28ºC
CBMAI1320	*Staphylococcus warneri/pasteuri* SG 52	JQ183026	13	P5	TSA, 28ºC
CBMAI 982	*Bacillus Vasafensis* SG 54_1	GQ272682 JQ183059	9	AF	NA, 28ºC
CBMAI 983	*Bacillus safensis* SG 54_2	GQ272683	9	AF	NA, 28ºC
CBMAI1297	*Bacillus safensis* SG 55	[a]	9	AF	NA, 28ºC

CBMAI 984	*Bacillus safensis* SG 56	GQ272684	5	AF	NA, 28ºC
CBMAI1298	*Bacillus* sp. SG 57	JQ183023 JQ183058	11	AF	NA, 28ºC
CBMAI1299	*Bacillus safensis* SG 58_1	a	9	AF	NA, 28ºC
CBMAI 985	*Bacillus safensis* SG 58_2	GQ272685	9	AF	NA, 28ºC
CBMAI1322	*Stenotrophomonas maltophilia* SG 59	JQ183018	4	P5	NA, 28ºC
CBMAI 986	*Bacillus safensis* SG 61	GQ272686	7	AF	NA, 28ºC
CBMAI1300	*Bacillus safensis* SG 62	a	5	AF	NA, 28ºC
CBMAI1277	*Bacillus thuringiensis*SG_C	JQ183024	12	P2	NA, 28ºC
CBMAI1316	*Bacillus firmus* P1_1	JQ183033	24	P1	TSA, 35ºC
CBMAI1383	*Bacillus megaterium* P1_4	JQ183034	25	P1	TSA, 28ºC
CBMAI1276	*Bacillus thuringiensis*P2_1	JQ183031	12	P2	MA, 35ºC
CBMAI1380	*Bacillus megaterium* P2_2	a	25	P2	TSA, 50ºC
CBMAI1365	*Micrococcus luteus* P4_1	JQ183041	18	P4	MA, 35ºC
CBMAI1379	*Micrococcus luteus* P4_3	JQ183040	18	P4	TSA, 35ºC
CBMAI1366	*Micrococcus luteus* P4_4	a	18	P4	MA, 35ºC
CBMAI1399	*Micrococcus luteus* P4_5	a	18	P4	GYM, 35ºC
CBMAI1370	*Staphylococcus warneri/ pasteuri* P5_3	JQ183036	13	P5	TSA, 28ºC
CBMAI1381	*Halomonas shengliensis* AF 5	JQ183046	27	AF	TSA, 35ºC
CBMAI1382	*Halomonas shengliensis* AF 6	a	27	AF	TSA, 35ºC
CBMAI1367	*Marinobacter lutaoensis*AF8	a	21	AF	TSA, 35ºC
CBMAI1368	*Marinobacter lutaoensis* AF9	JQ183045	22	AF	MA, 35ºC
CBMAI1369	*Staphylococcus warneri/ pasteuri* AF10	a	13	AF	MA, 35ºC
CBMAI1371	*Marinobacter lutaoensis*AF 12	a	21	AF	TSA, 35ºC
CBMAI1384	*Marinobacter lutaoensis*AF13	JQ183043	21	AF	TSA, 35ºC
CBMAI1385	*Halomonas shengliensis* AF14	JQ183047	27	AF	TSA, 35ºC
CBMAI1372	*Micrococcus luteus* AF16	JQ183042	18	AF	TSA 35ºC
CBMAI1386	*Halomonas shengliensis* AF17	JQ183048	27	AF	TSA, 28ºC
CBMAI1387	*Halomonas shengliensis* AF18	a	27	AF	MA, 28ºC
CBMAI1388	*Halomonas shengliensis* AF19	a	27	AF	MA, 35ºC
CBMAI1378	*Marinobacter lutaoensis*AF 20	JQ183029	16	AF	MA, 35ºC
CBMAI1373	*Marinobacter lutaoensis*AF21	JQ183044	21	AF	MA,35ºC
CBMAI1397	*Marinobacter lutaoensis*AF26	a	21	AF	MA, 35ºC
CBMAI1389	*Marinobacter lutaoensis*AF27	a	21	AF	MA, 35ºC
CBMAI1390	*Halomonas shengliensis* AF28	JQ183049	28	AF	MA, 50ºC
CBMAI1391	*Halomonas shengliensis* AF 29	JQ183016	1	AF	MA, 50ºC
CBMAI1392	*Halomonas shengliensis* AF30	a	27	AF	MA, 28ºC
CBMAI1374	*Staphylococcus hominis* AF32	JQ183035	13	AF	MA, 35ºC
CBMAI1375	*Marinobacter lutaoensis*AF33	a	21	AF	MA, 35ºC
CBMAI1393	*Halomonas shengliensis* AF34	a	27	AF	MA, 35ºC
CBMAI1275	*Bacillus pumilus*AF35	JQ183032 JQ183054	26	AF	MA, 35ºC
CBMAI1394	*Marinobacter lutaoensis*AF39	a	21	AF	MA, 35ºC
CBMAI1395	*Marinobacter lutaoensis*AF40	a	16	AF	MA, 50ºC
CBMAI1400	*Streptomyces alboniger/ chartreusis/moderatus* AF43	JQ183037	23	AF	TSA, 35ºC
CBMAI1401	*Kocuria rosea* AF44	JQ183039	19	AF	GYM, 28ºC
CBMAI1376	*Streptomyces alboniger/ chartreusis/moderatus* AF45	JQ183038	20	AF	GYM, 28ºC
CBMAI1396	*Marinobacter lutaoensis*AF 46	JQ183030	17	AF	GYM, 28ºC
CBMAI1377	*Citreicella thiooxidans* AF47	JQ183050	29	AF	MA, 35ºC

aBacteria were not sequenced, identification was achieved by comparing ARDRA profiles.
bOrigin of strain: AF-Formation Water; P1–Oil well 1; P2–Oil Well 2; P3–Oil Well 3; P4–Oil Well 4; P5 Oil Well 5.

Table 1: Bacterial strains isolated from petroleum formation water and oil samples, deposit number at CBMAI, genbank access numbers, ARDRA profiles, origin of strains, culture media and incubation temperature.

the Kit DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBace DNA Analysis Systems (GE Healthcare), according to the manufacturer´s specifications. Primers used for sequencing were 10F, 1100R [32] and 782R [36]. Sequencing of the fragments corresponding to the gyrase partial gene was performed using the primers UP-1 and UP-2r [34].

Partial gene sequences, 16S rRNA or gyrase, obtained with each

primer were assembled into a contig using phred/Phrap/CONSED program [37,38]. Identification was achieved by comparing the contiguous 16S rRNA or gyrase sequences obtained with sequence data from reference and type strains available in the public databases GenBank (www.ncbi.nlm.nih.gov) and RDP (Ribosomal Database Project-Release 10; http://rdp.cme.msu.edu/). The sequences were aligned using the CLUSTAL X program [39] and analyzed with MEGA software v.4 [40]. Evolutionary distances were derived from sequence-pair dissimilarities calculated as implemented in MEGA, using Kimura's DNA substitution model [41]. The phylogenetic reconstruction was done using the neighbor-joining (NJ) algorithm [42], with bootstrap values calculated from 1000 replicate runs.

Nucleotide sequence accession numbers

Sequences determined in this study were deposited at the Genbank database under the accession numbers JQ183016 to JQ183050 for the 16S rRNA gene sequences, and JQ183051 to JQ183059 for the gyrase gene sequences, both listed in table 1.

RAPD analysis

The molecular technique RAPD (Random Amplified Polymorphic DNA) was employed in order to genetically differentiate the bacterial isolates belonging to the same species. Three of the six primers listed below were used for typing the bacterial strains in RAPD independent reactions: UBC # 12 (5´-CCT GGG TCC A-3´), UBC # 25 (5´-ACA GGG CTC A-3´), UBC # 31 (5´-CCG GCC TTC C-3´), UBC # 2 (5´-CCT GGG CTT G-3´), UBC # 4 (5´-CCT GGG CTG G-3´) and UBC # 15 (5´-CCTGGGTTTG-3´) (Set 100/1; University of British Columbia, Vancouver, Canada).

Twenty five μl reaction mixtures contained 5 ng of genomic DNA, 2 U of Taq DNA polymerase (Invitrogen), 1X Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mix (GE Healthcare) and 1 mM primer. The amplification program consisted of one cycle at 95ºC for 2 min, 30 cycles of 30 seconds at 94ºC, 30 seconds at 36ºC and 1 minutes at 72ºC and final extension cycle at 72ºC for 3 minutes. The primers used were selected among those producing the most polymorphic profiles in a preliminary screening. The RAPD products were subjected to electrophoresis at 100 V for 2 h 30 min in 1.5 % agarose gel stained with ethidium bromide.

The gel images obtained with ARDRA and RAPD techniques were analyzed using the program Gelcompar 4.1 (Applied Maths, Kortrijk, Bélgica). Gels were normalized and UPGMA-based dendrograms constructed from Pearson (product-moment) correlation coefficient matrices [43]. The value of ≥70 % similarity was used as the cut off for the establishment of ARDRA clusters. The consistency of the clusters as representatives of different taxonomic groups was confirmed by sequencing and phylogenetic analysis of some isolates. For the analysis of the genetic fingerprints obtained by RAPD, the value of ≥75 to 80% similarity was used as the cut off for the genetically identical isolates [44].

Biodegradation assays

The bacterial strains were firstly cultured in Erlenmeyer flasks (1000 ml) containing 400mlof the same medium used for the isolation and at the appropriate temperature (Table 1). After 48 h of incubation on a rotational shaker (150 rpm), the biomass was recovered by centrifugation (11.5 g, 18ºC, and 20 min). The supernatant was discarded and the pellet (ca. 0.2 g) transferred to Erlenmeyer flasks

(125 μl) containing 40 ml Zinder medium [45] for the subsequent biodegradation assays.

Nonadecanoic acid (Aldrich), 4-cholesten-3-one (Aldrich), squalane (Aldrich). 9,10-dihydrophenanthrene (Aldrich) and nonadecane (Aldrich)were applied as substrates for the microbial growth and representatives of different classes of Brazilian petroleum biomarkers. Each compound (0.010 g) was prepared as previously described by Vasconcellos et al. [21]. Briefly, they were homogenized using tween 80 (300 μl), absolute ethanol (300 μl) and ethyl acetate (400 μl) as eluents. The final concentration of each biomarker in the biodegradation assays was 0.25 mg/ml. Negative controls were also developed, consisting of: (i) Zinder mineral medium added by the solubilized biomarkers; (ii) mineral medium plus biomarker solution and bacterial dead cells (autoclaved); (iii) solely mineral medium; (iv) inoculum of the bacterial strain in the mineral medium containing the eluents without biomarkers [21].

The flasks were inoculated in triplicate for each time sampled (0, 7, 14, 21 and 28 days) and incubated on a rotational shaker at 150 rpm. The production of microbial metabolites and the biomarkers degradation were monitored by GC–MS analysis. The biodegradation ability of the isolates was monitored using the biomarkers in mixtures (named here as biomarker cocktail).

After growth, the total culture volume of each assay, including the controls, were transferred to glass vials and submitted to organic extraction (2X) using ethyl acetate (20 ml) as eluent. The vials were vortexed and the organic phase was transferred to other vials. The organic extracts were derivatized by methylation reaction according method described by Kosak et al. [46] followed by drying at N_2 flux. The dried extracts were diluted in ethyl acetate (995 μl) and heptadecane solution (5 μl, 0.03 mg/ml), used as internal standard in the GC–MS analysis.

GC-MS analysis were performed using a HP6890II instrument coupled to a mass detector HP5970-MSD, equipped with a HP-5-MS fused silica column (30 m×0.25 mm, 0.25 μm film thickness). He was the carrier gas (ca. 1 ml min⁻¹) and the oven temperature program was 60–290°C (held 5 min) at 10°C min⁻¹. The instrument was operated in the SCAN mode over a range of m/z 50–700. The injector (240°C) was operated in the split mode (10:1). The extent of biodegradation was measured from the chromatographic data, applying the equations based on calculations described by Aldas et al. [47], and detailed by Vasconcellos et al. [21].

Results

ARDRA, sequencing and phylogenetic analysis

Ninety eight strains of aerobic organotrophic bacteria isolated from oil and formation water samples were differentiated by macroscopic (colony color, form and size) and microscopic (cell morphology and Gram-staining) characteristics (data not shown). Bacterial isolates were subsequently subjected to ribotyping by ARDRA in order to select band patterns (ribotypes) for subsequent phylogenetic affiliation. Screening by ARDRA with 3 different enzymes allowed the detection of 28 bacterial ribotypes representing potentially different taxonomic groups (Table 1). In total, 39 bacteria were selected for further identification via molecular taxonomy, based on partial sequencing and phylogenetic analysis of 16S rRNA genes. Identification of the remaining 59 bacterial isolates was based on the comparison of their ribotypes with the ones of the sequenced strains (Table 1).

Phylogenetic analysis of 16S rDNA sequences of the isolates revealed

that ribotypes represented, in fact, 16 different taxonomic groups. Additionally, phylogenetic analysis allowed for the identification of many bacterial members at the species level (Figure 1-3). Isolates P1_1, P1_4, SG23_2 and SG25 clustered, respectively, with *Bacillus firmus*, *Bacillus megaterium*, *Bacillus simplex* and *Bacillus sphaericus*, with high bootstrap values (97-99%)

On the other hand, some bacterial isolates could not be identified at the species level due to the conserved nature of the 16S rRNA gene of the related species. This was the case of the isolates P5_3 and SG52, which clustered with *Staphylococcus pasteuri* and *Staphylococcus warneri* (Figure 1), and the isolates AF46 and AF45, which grouped with *Streptomyces chartreusis, Streptomyces moderatus* and *Streptomyces alboniger* (Figure 3).

In addition, isolates SG1, SG5, SG10, SG47_2, SG54_1, SG57 and AF35 clustered, with a high bootstrap value (99%), with *Bacillus pumilus* and *B. safensis* strains, including the type strains of suchspecies (Figure 1). And finally, this was also observed for the isolates SG21 and P2_1, that grouped with *Bacillus cereus* and *Bacillus thuringiensis* 16S rRNA sequences (99% boostrap value). For the *Bacillus*-related isolates, the gyrase gene (*gyr*) was used as an alternative phylogenetic marker to allow for the identification at the species level. Phylogenetic reconstruction based on the gyrase gene revealed that isolatesSG1, SG5, SG10, SG12_2, SG30 and SG54_1grouped with *Bacillus safensis* (70-90% boostrap value) and AF35 with *Bacillus pumilus* (100% boostrap value) Figure 4.

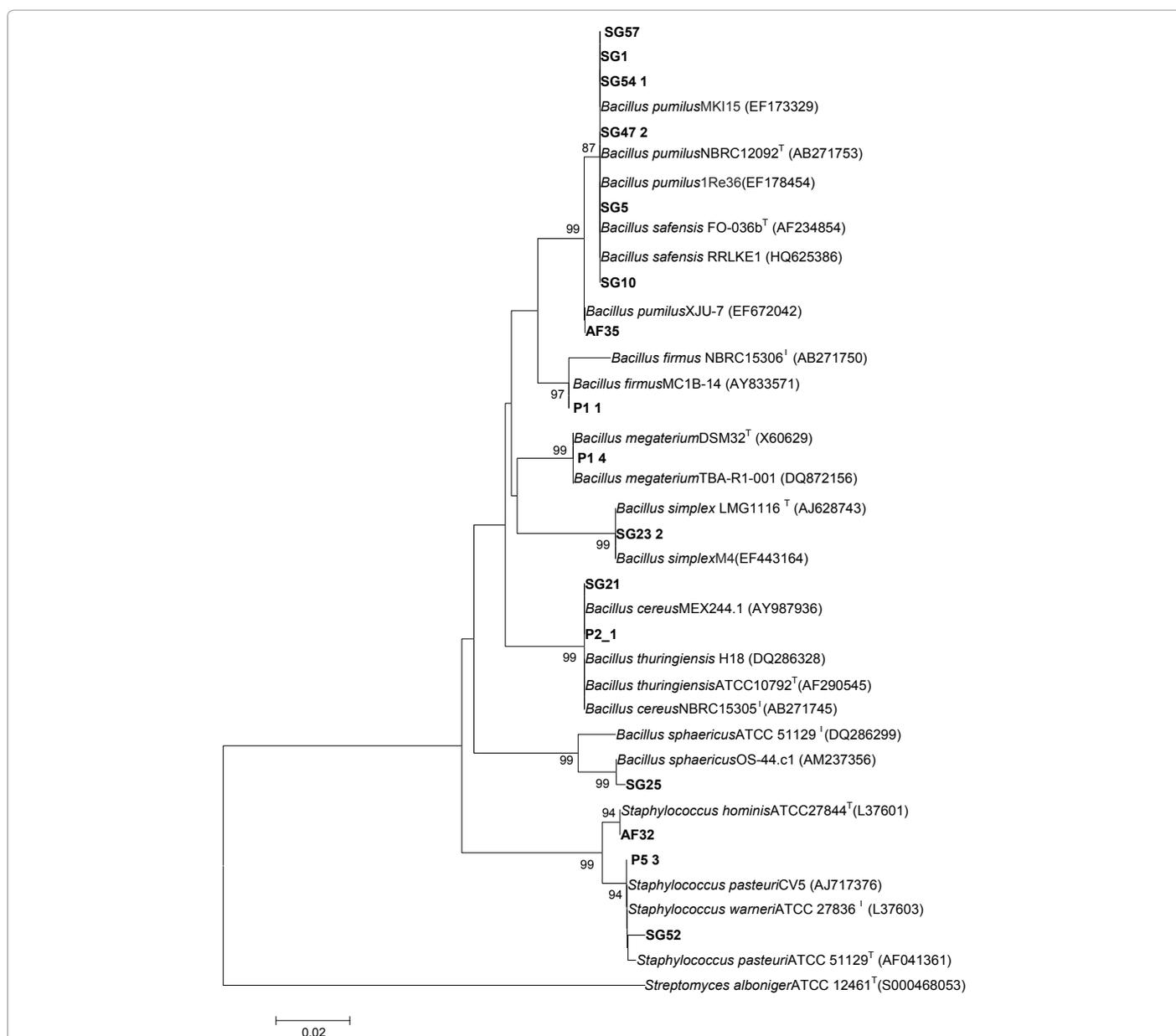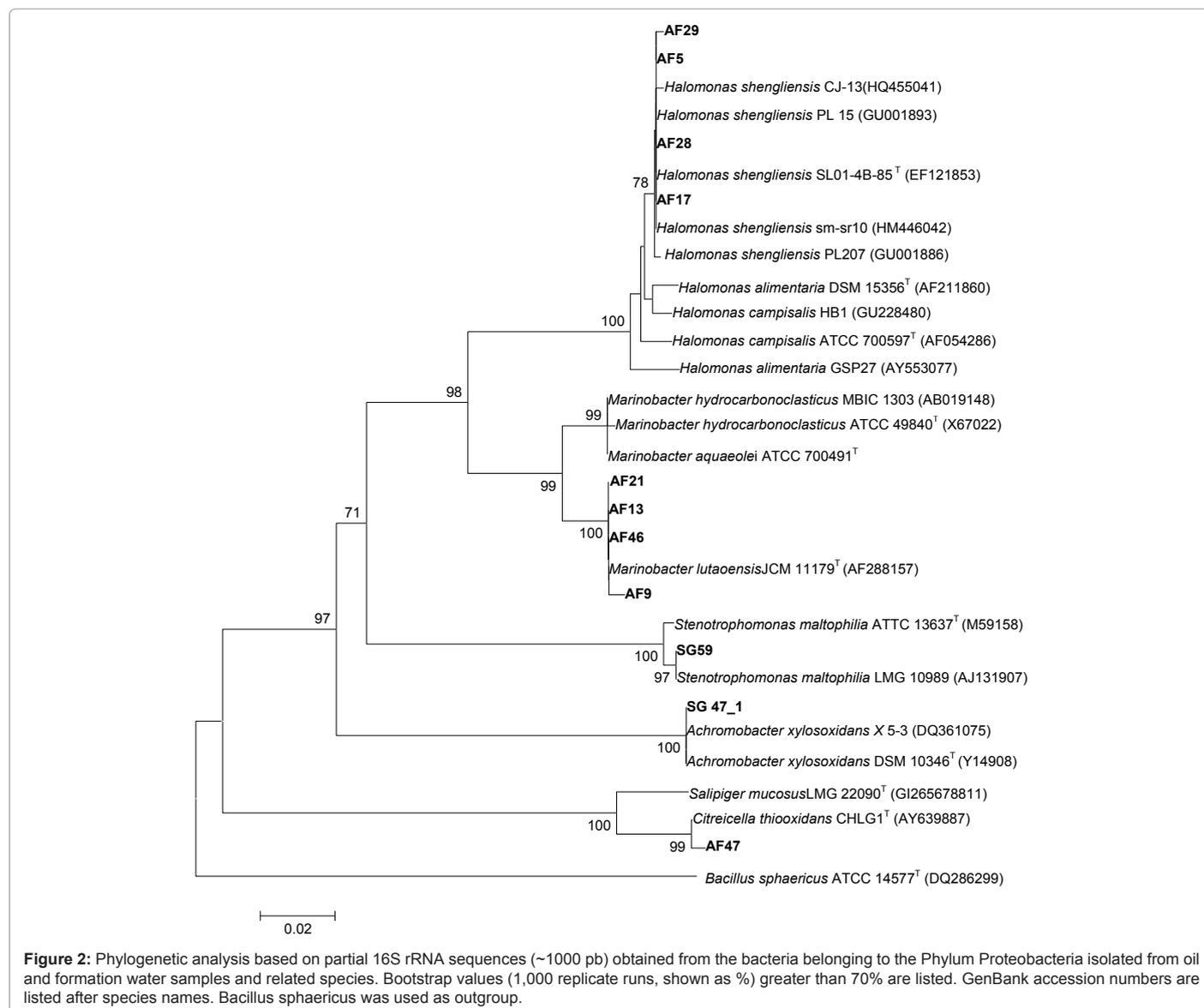SG47_2 and SG57 grouped with each other in a separate cluster

Figure 1: Phylogenetic analysis based on partial 16S rRNA sequences (~1000 pb) obtained from the bacteria belonging to the Phylum Firmicutes isolated from oil and formation water samples and related species. Bootstrap values (1,000 replicate runs, shown as %) greater than 70% are listed. GenBank accession numbers are listed after species names. *Streptomyces alboniger* was used as outgroup.

Figure 2: Phylogenetic analysis based on partial 16S rRNA sequences (~1000 pb) obtained from the bacteria belonging to the Phylum Proteobacteria isolated from oil and formation water samples and related species. Bootstrap values (1,000 replicate runs, shown as %) greater than 70% are listed. GenBank accession numbers are listed after species names. Bacillus sphaericus was used as outgroup.

supported by100% boostrap value. Although they were shown to be more closely related to *B. safensis*, the evolutionary distance observed in the phylogenetic tree suggests that they may represent a new *Bacillus* species.

Identification of isolates SG_C, SG15, SG20, SG21 and P2_1at the species level was achieved by specific PCR amplification using two sets of primers for the gyrase gene, one for *Bacillus cereus* and other for *Bacillus thuringiensis*. Amplification of the gyrase gene for these isolates was positive only when using the *Bacillus thuringiensis*primers (Figure 5).

RAPD

Isolates belonging to the same species were subjected to RAPD typing, using 3 primers in independent reactions, aiming at the differentiation at the infra-specific level. RAPD fingerprints allowed us to successfully discriminate the genetically distinct isolates.

Except for the species *Bacillus sphaericus* and *Stenotrophomonas maltophilia*, all the taxa under study presented more than one RAPD profile, revealing a great genetic diversity among the isolates recovered from the petroleum samples. Although the majority of the isolates recovered belonged to the *Bacillus safensis* species, actually only half of them represented genetically distinct isolates (Table 2).

Biodegradation of petroleum biomarkers

The potential of the isolated bacteria to biodegrade petroleum biomarkers was evaluated by GC-MS analysis. As a common profile of all the evaluated strains, it could be observed the preferential use of the acid biomarker as substrate. The same fact was also reported by Vasconcellos et al. [21] when evaluating aerobic bacteria isolated from the Campos Basin.

Biodegradation results revealed the ability of the bacterial strains obtained from the petroleum samples to degrade the evaluated hydrocarbonsin values up to89%.The comparison of the biodegradation values of the different hydrocarbons offered as substrates showed that the biomarker squalane was preferentially biodegraded by the majority of the strains. On the other hand, phenanthrene and nonadecane were
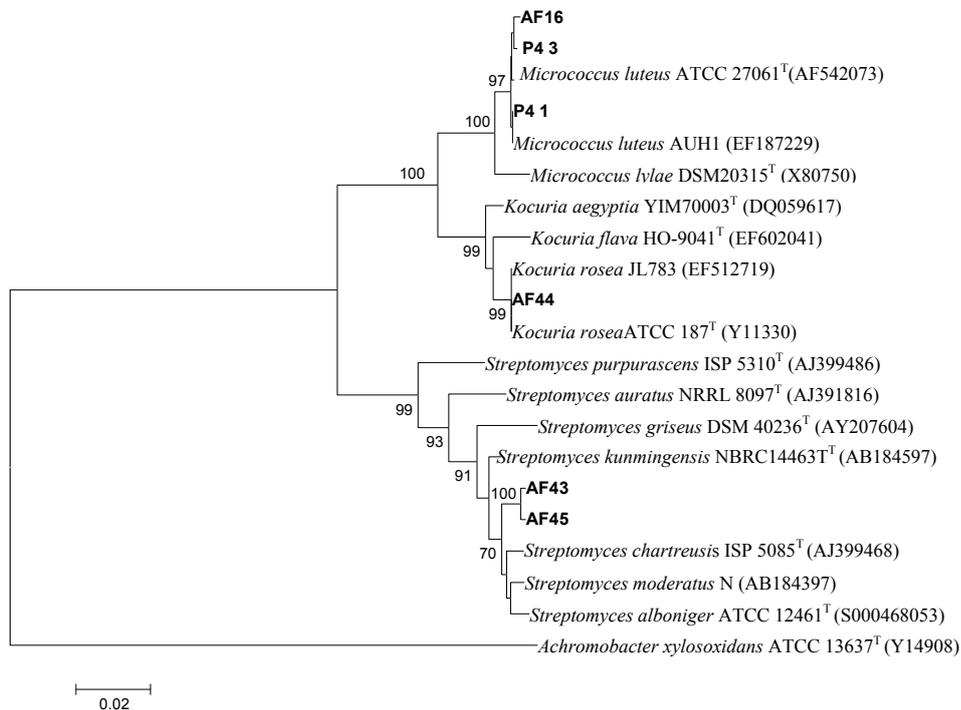
Figure 3: Phylogenetic analysis based on partial 16S rRNA sequences (~1000 pb) obtained from the bacteria belonging to the Phylum Actinobacteria isolated from oil and formation water samples and related species. Bootstrap values (1,000 replicate runs, shown as %) greater than 70% are listed. GenBank accession numbers are listed after species names. *Achromobacter xylosoxidans* was used as outgroup.
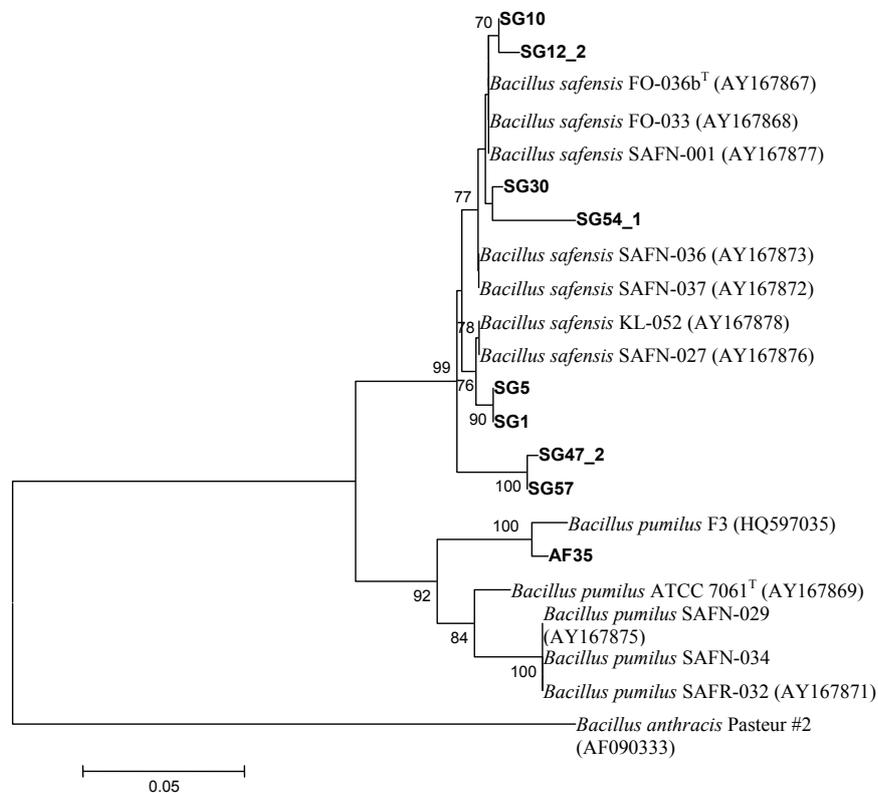

Figure 4: Phylogenetic analysis based on partial gyrase gene sequences obtained from the bacterial isolates belonging to the *Bacillus safensis*/*B. pumilus* group. Bootstrap values (1,000 replicate runs, shown as %) greater than 70% are listed. GenBank accession numbers are listed after species names. *Bacillus anthracis* was used as outgroup.
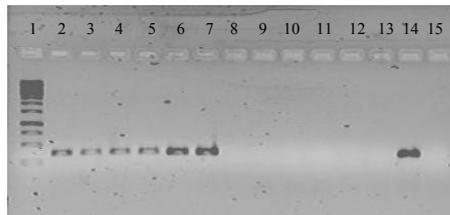
Figure 5: PCR amplification using gyrase gene-specific primer sets and genomic DNA from bacterial isolates belonging to the *Bacillus cereus/B. thuringiensis* group. (a) Lane 1, DNA molecular marker (100 bp DNA ladder, Fermentas); 2 to 8, isolates SGC, SG15, SG20, SG21, P2_1, positive and negative control, respectively, with primer set specific for *Bacillus thuringiensis*, 9 to 15, isolates SGC, SG15, SG20, SG21, P2_1, positive and negative control, respectively, with primer set specific for *Bacillus cereus*. Positive controls used for PCR were *Bacillus cereus* LFB-FIOCRUZ 406^T and *Bacillus thuringiensis* serovar israelensis LFB-FIOCRUZ 584, provided by Fiocruz.

Species	Number of isolates	Number of RAPD profiles
Bacillus safensis	48	23
Stenotrophomonas maltophilia	2	1
Bacillus thuringiensis	5	3
Bacillus sphaericus	2	1
Marinobacter lutaoensis	12	8
Bacillus megaterium	2	2
Staphylococcus warneri/S. pasteuri	3	3
Streptomyces chartreusis/S. moderatus	2	2
Micrococcus luteus	5	5
Halomonas shengliensis	10	5

Table 2: Summary of RAPD typing results of isolates belonging to the same species.

almost not biodegraded by any of the bacteria. *Bacillus thuringiensis* SG 21 biodegraded onlyphenanthrene and showed the highest biodegradation valuefor this biomarker (40%). The bacterial strains did not show biodegradation preference for the substrate 4-cholesten-3-oneas well (Table 3).

Discussion

The ARDRA methodology was an effective tool capable to group the isolates according to their ribotypes and to allow for the selection of only a few representatives of such ribotypes for further sequencing. However, 16S rRNA gene sequencing and phylogenetic analyses revealed that, in fact, some ARDRA groups corresponded to the same bacterial species, suggesting infra-specific variation in the 16S rRNA gene of *Stenotrophomonas maltophilia* (2 ribotypes), *Marinobacter lutaoensis* (2 ribotypes) *Halomonas shengliensis* (2 ribotypes) and *Bacillus safensis* (7 ribotypes).

Molecular taxonomy based on the 16S rRNA phylogenetic marker showed that the cultivated aerobic bacteria recovered belonged to 16 different species, distributed among 3 phyla: Firmicutes, Actinobacteria and Proteobacteria (classes Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria). Several genera of bacteria found in this study have already been detected in oil reservoirs, marine environments or associated with crude oil, such as *Marinobacter*, *Halomonas* [48,6], *Achromobacter*, *Streptomyces* [15], *Micrococcus luteus* [49], *Kocuria* [5], *Staphylococcus* and *Stenotrophomonas* [50], or have been commonly associated with the degradation of hydrocarbons, such as *Bacillus* [51]. The genera *Marinobacter*, *Halomonas*, *Kocuria*, *Staphylococcus* and *Streptomyces* have also been reported as hydrocarbon degraders of common occurrence in marine environments, [5,52-55].

The species *Bacillus safensis* was the most abundant cultivated bacteria recovered from the petroleum samples under study. These bacteria were isolated from all of the oil wells sampled, with different levels of biodegradation, depth and temperature, as well as from the formation water. In addition, the molecular typing method RAPD, used to differentiate the isolates at the infra-specific level, showed a high genetic diversity of *Bacillus safensis* in the samples. These data may suggest that *B. safensis* is adapted to the harsh conditions of petroleum reservoirs, i.e. high salinity, pressure and temperature and oxygen deficiency, indicating their ability to survive in extreme environments and their wide distribution in the Campos Basin reservoirs. This species was recently described by Satomi et al. [56] and is phylogenetically indistinguishable from *Bacillus pumilus* based solely on the 16S rRNA genetic marker, although this can be achieved on the basis of the gyrase gene. This was the first report on the occurrence of *Bacillus safensis* in petroleum-associated environments.

In this work, chromatographic analysis revealed that *B. safensis* strains SG 01, SG 30 and SG 32 were able to degrade 63% nonadecanoic acid and 13% nonadecane. The only strain of *Bacillus pumilus* found in this study, AF 35, showed degradation of all the biomarkers in the following percentages: 63% nonadecanoic acid, 79% squalane, 16% cholestenone, 15% phenanthrene and 9% nonadecane. Literature data have already reported one *B. pumilus* strain, isolated from an oil sample from Campos Basin, able to moderately biodegrade phytane (~40%) [21]. The same authors reported that *B. pumilus* populations could be broadly detected in biodegraded and non-biodegraded oil samples using a direct 5α-cholestane molecular approach based on group-specific PCR detection.

Other previous studies corroborate the isolation of *B. pumilus*, as well as other *Bacillus* species, from Brazilian petroleum reservoirs [57]. Literature data have already demonstrated the excellent ability of *B. pumilus* to degrade petroleum hydrocarbons [49,58,59] suggesting its potential to damage the oils sampled in this study.

Other species of the genus *Bacillus* identified in this work, such as *Bacillus sphaericus* and *Bacillusthuringiensis*, have already been found in environments associated with oil, such as crude oil storage tanks [50] and soil and freshwater lakes contaminated with oil [27,60] isolated strains of *Bacillus cereus* from the rock of an oil reservoir from a virgin field located in a deep-water production basin in Brazil, suggesting that these bacteria are autochthonous in these environments. However, *B. cereus* strains isolated in previous studies neither showed the ability to degrade petroleum hydrocarbons [27,60] nor presented PCR amplification of catabolic genes, such as n-alkane monooxigenase, catechol 1,2-dioxygenase and catechol 2,3-dioxigenase [27]. These data corroborate the results found in the present work, since some *Bacillus* spp. showed insignificant to moderate ability to degrade the biomarkers used in the GC-MS assays. These results may suggest that these bacteria are secondary degraders, i.e. they assimilate metabolites produced by the primary hydrocarbon biodegraders. As to the species *B. simplex*, no previous reports were found regarding the presence of this bacterium in marine or oil-related environments. In this study, *Bacillus simplex* strain SG23_2 was able to degrade 62% nonadecanoic acid and 79% squalane. On the other hand, the species *Bacillus firmus* has been isolated from different petroleum-associated environments, such as contaminated soils and oil reservoirs, and reported as being able to degrade polycyclic aromatic and aliphatic hydrocarbons [48,61]. Results obtained in the present study revealed significant nonadecanoic acid (63%) and squalane (68%) degradation extents by *B. firmus* strain P1_1, confirming literature data.

Strains	Biodegradation level of evaluated compounds (%)[a]				
	9,10-dihydrophe nanthrene	nonadecane	nonadecanoic acid	Squalane	Cholestenone
Bacillus fimus P1_1	-[b]	3	63	68	16
Micrococcus luteus P4_1	19	13	63	89	18
Staphylococcus hominis AF 32	20	13	-	89	18
Marinobacter lutaoensis AF 33	14	2	63	89	18
Halomonas shengliensis AF 19	9	13	63	89	18
Citreicella thiooxidans AF 47	14	2	63	78	18
Bacillus simplex SG 23_2	2	10	62	79	18
Bacillus megaterium P 2_2	4	-	62	-	7
Bacillus sphaericus SG 25	7	9	60	82	18
Bacillus pumilus AF 35	15	9	63	79	16
Stenotrophomonas maltophilia SG 59	10	0.2	63	73	18
Bacillus thuringiensis SG 21	40	-	-	-	-[b]
Achromobacter xylosoxidans SG 47_1	15	12	50	83	18
Kocuria rosea AF 44	17	11	63	79	18
Bacillus safensis SG 32	-	13	63	-	-
Streptomyces sp. AF 45	-	-	50	-	-
Bacillus safensis SG 01	-	13	63	-	-
Bacillus safensis SG 30	-	13	63	-	-

[a] net values (obtained by subtracting values determined for the negative control containing dead cells from the total biodegradation percentage).
-[b] biodegradation undetected.

Table 3: Biodegradation percentages of the petroleum biomarkers by the bacterial isolates.

Bacillus megaterium was also identified in this work and has already been reported in previous studies of bacterial isolation and biodegradation from petroleum environments [62]. The ability of these bacteria to biodegrade petroleum hydrocarbons has been broadly demonstrated elsewhere [63,64]. In this study, *Bacillus megaterium* strainP2_2 degraded preferentially nonadecanoic acid (62%), in comparison to the other evaluated biomarkers.

Achromobacter xylosoxidans strain SG 47_1 was recovered from the non-biodegraded oil reservoir 1. Populations of *A. xylosoxidans* were previously detected in samples from reservoirs 3 and 5 using a direct molecular approach based on group-specific PCR [21]. Results found by these authors showed that *A. xylosoxidans* had the highest cholestane degradation index (89%), in addition to the ability of degrading phytane and nonadecanoic acid. Another study conducted by our research group reported the isolation of *Achromobacter xylosoxidans*from oil samples collected from one reservoir with a high level of biodegradation and temperature of 52°C [15]. These data may reflect the ability of this bacterium to survive under conditions of high pressure and temperatures between 52 and 85°C. This species has been isolated from diverse environments associated with oil other than oil reservoirs, such as refinery wastewater treatment plants [65] and crude oil storage tanks [50]. Several literature data have demonstrated the ability of *A. xylosoxidans*to degrade mono and polyaromatic petroleum hydrocarbons [66-68]. In the present work, *A. xylosoxidans* strain SG47_1 exhibited more significant biodegradation percentages for the nonadecanoic acid (50%) and squalane (83%), confirming its potential to biodegrade not only long chain acid but also branched hydrocarbon.

One of the strains identified in this study showed high 16S rRNA sequence similarity with *Stenotrophomonas maltophilia* and it could degrade 63% of nonadecanoic acid and 73% of the squalane. This species has been previously described as isolated from stored crude oil, with an excellent ability to grow on medium containing *n*-eicosane (C20) as sole carbon source, suggesting its potential to degrade petroleum compounds [50].

Bacteria belonging to the genus *Marinobacter* were also identified among the strains under study. Our results corroborate previous literature data on the detection of *Marinobacter hydrocarbonoclasticus* [6] and *Marinobacter lipolyticus* [15] in samples from oil reservoirs by using cultivation-independent methods. *M. hydrocarbonoclasticus* is an extremely halotolerant marine bacterium and able to degrade hydrocarbons [69,70,52], whereas *M. lipolyticus* is a halophilic species with lipolytic activity, originally isolated from hypersaline environments [71]. The strain identified in the present study was closely related with the species *Marinobacter lutaoensis*, which is a thermotolerant bacterium described from a hot spring on the coast of Taiwan [72]. The evaluation of the biodegradation results obtained for *Marinobacter lutaoensis* AF33 showed a preferential consumption of squalane (89%), followed by nonadecanoic acid (63%), which confirm the same profiles obtained for the other evaluated strains in this study.

Two strains of *Streptomyces* spp. were recovered from the formation water samples, and although this genus is not frequent and/or predominant in petroleum reservoirs, some representatives may be found in environments associated with oil or related to the degradation of hydrocarbons [6,73,74]. Representatives of this genus have been also detected in degraded and non-degraded oil samples from the Campos Basin by using 16S rDNA libraries [15]. The authors found a higher abundance of these populations in a non-biodegraded oil sample, suggesting that these organisms are not primarily responsible for degradation of hydrocarbons in these reserves. *Streptomyces* spp. were also reported by Vasconcellos et al. [21], using group-specific PCR. *Streptomyces* spp. strain AF 45 isolated in the present study was also evaluated in the biodegradation assays. It showed preferential biodegradation of nonadecanoic acid (50%), not presenting ability for the consumption of hydrocarbons as carbon sources.

Ten strains isolated from formation water showed close phylogenetic relationship *Halomonas shengliensis* [75], which was recently described as a new species of moderately halophilic bacteria isolated from saline soil, contaminated with crude oil in the coastal

Shengli oil field in China. In a study of bacterial communities using culture-independent methods, the authors also could found *Halomonas* spp. in production water samples from reservoirs in California [6]. As observed for the majority of the bacterial isolates in this study, *Halomonas shengliensis* AF 19 showed the high biodegradation values and preferential consume of nonadecanoic acid (63%) and squalane (89%) as substrate for its microbial growth.

Staphylococcus hominis and *Kocuria rosea* have been previously found in soil contaminated with diesel oil obtained from an underground leak of a gas station [76]. The same authors described that these bacteria were the most prevalent among those isolated from the consortia, and they are able to accelerate the removal of petroleum hydrocarbons. The genus *Kocuria* was also reported in another phylogenetic diversity study of aerobic bacteria isolated from formation water of a Chinese reservoir and its ability to use petroleum hydrocarbons was also confirmed [5]. In our study, the strain AF 44 phylogenetically identified as *Kocuria rosea* followed the degradation pattern of the most bacterial isolates, with highest percentages of biodegradation for nonadecanoic acid (63%) and squalane (79%). Differently, *Staphylococcus hominis* strain AF 32 did not show ability to biodegrade nonadecanoic acid, but it was able to degrade preferentially squalane (89%).

The species *Micrococcus luteus*, recovered in this study from the formation water and from the highly biodegraded oil from reservoir 4, has been reported in several studies as an efficient degrader of hydrocarbons, including naphthalene and phenanthrene [49,77,78]. In a previous study Vasconcellos et al. [21] found populations of *Micrococcus* spp. in oil reservoirs 1, 3, 4 and 5 and formation water from an oil field in the Campos Basin, using a direct molecular approach based on group-specific PCR. These authors also demonstrated that these bacteria have great capacity for biodegrading isoprenoids (phytane) and aromatics (dihydrophenanthrene), suggesting their potential as a spoiling agent in oil reservoirs. In the present work, *M. luteus* strain P4_1 showed better results for the biodegradation of nonadecanoic acid (63%) and squalane (89%).

Citreicella thiooxidans, a new genus and species described by Sorokin et al. [59], was also identified from samples of petroleum formation water. These bacteria were first isolated from Black Sea and they depend of NaCl for growth. They have also ability to oxidize thiosulfate, sulfide and sulfur to sulfate, using the metabolic energy of these reactions for growth [79]. *Citreicella thiooxidans* strain AF 47 was also found in our study, and it showed similar biodegradation profiles to the other isolates described, exhibiting preference for the degradation of nonadecanoic acid (64%) and squalane (78%).

In general, the results of the biodegradation assays revealed the preference of the bacterial isolates for the degradation of the nonadecanoic acid. This was also observed in previous studies of our research group for some *Bacillus* strains [21] and corroborates literature data on production and use of carboxylic acids during the aerobic microbial metabolism [80].

The evaluated strains revealed significant biodegradation values (up to 89%) for squalane, revealing a potential ability to biodegrade isoprenoid biomarkers. Nonadecane showed lower percentages of degradation and the nonadecanoic acid showed percentages around 60% for most of the evaluated bacteria, except for *Bacillus thuringiensis* and *Staphylococcus hominis*.

A similar profile was described by Bogan et al. [81] in a study where *Alkanindiges illinoisensis*, an obligatory hydrocarbon degrading bacterium, was isolated from a soil of an oilfield in Southern Illinois.

The authors reported that this strain could only grow when the medium was added of hydrocarbons, especially squalane. Radwan et al. [82] also reported about a bacterial strain, *Arthrobacter nicotianae* KCC B35, able to grow on hydrocarbons with chain lengths of C16 or longer. As it was found in the present work, Cabezali and collaborators isolated some strains able to hydrocarbon degradation, and one of these was capable to grow on linear alkanes and fatty acids [83.84].

In addition, the bacteria *Mycobacterium fortuitum* and *Mycobacterium ratisbonense*, isolated from a sewage treatment plant, were shown to be capable of utilizing the multiply branched hydrocarbon squalane and its analogous unsaturated hydrocarbon squalene as the sole carbon source for growth [85]. The authors evaluated the growth of both strains on alkanes, acyclic isoprenoids, and acids derived from these compounds in order to elucidate the pathway for the degradation of squalane by these bacteria. The results obtained allowed them to propose a putative pathway where, after the conversion of squalane to a dioic acid as one of the first intermediates, three propionyl coenzyme A and acetyl coenzyme A molecules are oxidatively removed by the β-oxidation route to form the 3,7,11-trimethyldodecandioic acid intermediate by a pathway analogous to that for the degradation of the multiply branched alkane pristine (2,3,10,14-tetramethylpentadecane). Finally, a β-methyl group of this intermediate can be converted into a carbonyl oxygen, thus generating a suitable substrate for β-oxidation and further degradation. These investigations could be important to develop a new approach to solve specific bioaccumulation problems associated with alkyl-branched compounds, like squalane, which confer molecular recalcitrance [85]. Thus, the present work allowed the recovery of bacterial strains with ability and preference to squalane biodegradation, corroborating previous literature data and opening the perspective for future application in biotechnological processes.

Although cultivation techniques have been improved and have led to the in vitro recovery of a growing number of yet uncultivated microorganisms [86], our knowledge on their ecology remains insufficient to grow the most of them. This is particularly true for microbial communities that degrade recalcitrant compounds or pollutants, where the complexity of the metabolic processes required for this degradation leads to the formation of consortia containing bacteria of different genera and species; each one specialized in degrading one or more compounds [87]. In this context, the bacterial species recovered in the present study represent, in fact, only a small fraction of the total microbiota present in the environment of the oil reservoir, as already observed for many other natural environments [88]. Thus, possibly, many other species responsible for the in situ degradation of oil compounds were not recovered in this study, making clear the limitations imposed by the use of pure cultures in biodegradation studies.

Nonetheless, the results gathered in this study certainly contribute to the knowledge of the phylogenetic diversity of bacteria recovered from oil reservoirs, offering a great potential for basic research and technological exploitation.

Acknowledgments

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