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Bioremediation potential of microorganisms derived from petroleum reservoirs

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ABSTRACT

Bacterial strains and metagenomic clones, both obtained from petroleum reservoirs, were evaluated for petroleum degradation abilities either individually or in pools using seawater microcosms for 21 days. Gas Chromatography–Flame Ionization Detector (GC–FID) and Gas Chromatography-Mass Spectrometry (GC–MS) analyses were carried out to evaluate crude oil degradation. The results showed that metagenomic clones 1A and 2B were able to biodegrade n-alkanes (C14 to C33) and isoprenoids (phytane and pristane), with rates ranging from 31% to 47%, respectively. The bacteria *Dietzia maris* CBMAI 705 and *Micrococcus* sp. CBMAI 636 showed higher rates reaching 99% after 21 days. The metagenomic clone pool biodegraded these compounds at rates ranging from 11% to 45%. Regarding aromatic compound biodegradation, metagenomic clones 2B and 10A were able to biodegrade up to 94% of phenanthrene and methylphenanthrenes (3-MP, 2-MP, 9-MP and 1-MP) with rates ranging from 55% to 70% after 21 days, while the bacteria *Dietzia maris* CBMAI 705 and *Micrococcus* sp. CBMAI 636 were able to biodegrade 63% and up to 99% of phenanthrene, respectively, and methylphenanthrenes (3-MP, 2-MP, 9-MP and 1-MP) with rates ranging from 23% to 99% after 21 days. In this work, isolated strains as well as metagenomic clones were capable of degrading several petroleum compounds, revealing an innovative strategy and a great potential for further biotechnological and bioremediation applications.

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1. Introduction

Oil spills are a worldwide problem, drastically affecting marine ecosystems and, indirectly, the human populations that depend on marine resources. Oil spills occur through accidental leakage at petroleum production facilities or as a consequence of the petroleum processing industry (Mei and Yin, 2009). There are many reports describing oil spills all over the world, including Prestige in 2002 in Spain and the 2010 accident involving the petroleum company "British Petroleum" (Deep Water Horizon spill) in the Gulf of Mexico. Recently, in Brazil, a significant oil spill occurred in 2011 in Campos Basin, involving the company "Chevron", which resulted in the release of approximately 3000 petroleum barrels into the ocean (Duarte et al., 2013; Cameron, 2012).

In the water, the oil forms slicks over the sea surface causing limited gas exchange through the air-sea interface and reducing light penetration into the water column, affecting phytoplankton photosynthesis (González et al., 2009). In addition, tar balls are deposited on beaches and mangroves (Mulabagal et al., 2013). The toxic effects of petroleum in marine ecosystems have been studied for years, including damage caused to algae and phytoplankton, which represent the primary level of the marine food chain (Durako et al., 1993; Carrera-Martínez et al., 2010). The hydrocarbons from petroleum may enter the food chain and accumulate in biological tissue, representing an intoxication risk for all individuals involved (Perelo, 2010) In addition, many compounds present in petroleum, such as polycyclic aromatic hydrocarbons (PAHs), are recalcitrant and may persist for long periods of time in the environment.

Bioremediation is a technique that employs living organisms aimed at the mineralization of pollutants, resulting in the removal or attenuation of the pollutant compound to a less harmful product in the contaminated area. Bioremediation can be performed by

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bacteria, yeasts or fungi (Luqueño et al., 2011; Bamforth and Singleton, 2005). Biostimulation and bioaugmentation are distinct strategies used to treat impacted environments. However, both are considered to be forms of bioremediation. Biostimulation involves nutrient addition, such as nitrogen or phosphorus, to stimulate the growth of the indigenous microbial community and improve the biodegradation process. When the pollutant is poorly soluble in water, emulsifiers or surface-active agents are often added to enhance solubility and, thus, degradation (Calvo et al., 2009). Biostimulation has been widely studied, mainly for *in situ* application where it is possible to observe changes in the microbial patterns of the indigenous community (Cappello et al., 2007; Evans et al., 2004). Bioaugmentation is a technique that improves the degrading potential of contaminated areas through the introduction of specific microbial strains or consortia known to be efficient degraders. However, the efficiency of bioaugmentation is determined by many abiotic and biotic factors, such as chemical structure, concentration and availability of pollutants in the contaminated environment, and environmental temperature. In addition, the selection of appropriate microorganisms is a key factor that requires the monitoring of several variables that may affect the success of the bioremediation process, including the capacity to degrade contaminants, competition with autochthonous microorganisms, predation by protozoa, loss of microbial viability and eventual cell death after inoculation (Mrozik and Seget, 2010; Tyagi et al., 2010).

Petroleum is a complex mixture of hydrocarbons and non-hydrocarbons (metal porphyrins, acid and organometallic compounds) (van Hamme et al., 2003). Fortunately, several microorganisms are equipped with metabolic machinery to use petroleum as a carbon and energy source through either aerobic or anaerobic pathways (Magot et al., 2000; Borzenkov et al., 2006; da Cruz et al., 2011). *n*-Alkanes, isoprenoids, hopanes, steranes and aromatic hydrocarbons are biomarker compounds found in crude oil from formerly living organisms whose organic materials were preserved within source rocks over geologic times (Peters et al., 2005; Wang and Yang, 2007).

Biomarker diagnostic parameters have long been established and are widely used by geochemists for evaluation of oil biodegradation in-reservoir (Peters et al., 2005) and simulated experiments (Da Cruz et al., 2011; Bao et al., 2014). For example, the ratios of pristane/n-C17 (Pr/n-C17) and phytane/n-C18 (Ph/n-C18) have been shown to be reliable indicators of biodegradation (e.g.,Winters and Williams, 1969) because the n-alkanes are always present in lower relative concentrations than isoprenoids in biodegraded oils (Asif et al., 2009). However, these ratios may underestimate the extent of biodegradation because Pr and Ph could also be degraded under severe weathering conditions over a long period of time (Bao et al., 2014). Carbon preference index (CPI), obtained from the distribution of n-alkanes, is affected by both the source and maturity of crude oils (Tissot and Welte, 1984) and also can be used to monitor the susceptibility of odd and even carbon-numbered n-alkanes to degradation (Peters and Moldowan, 1993; Venosa et al., 1997; Bao et al., 2014).

Hydrocarbon-degrading microorganisms encompass a long list of genera, including *Bacillus, Brevibacillus, Pseudomonas, Acinetobacter, Dietzia, Methylobacterium,* and *Rhodococcus,* among others (von der Weid et al., 2007; da Cruz et al., 2010; Verde et al., 2013; Pacheco et al., 2010). In this scenario, bioremediation has become a suitable alternative, and microbial blends and biosurfactants are already commercialized by some companies around the world (Banat et al., 2010; Zhu et al., 2004). Some of these substances have been evaluated in scientific works described in the literature and have been proven to be effective when used as a biostimulation approach (Silva et al., 2009; Mohammed et al., 2007). However, in addition to examining petroleum degradation abilities, it is necessary to evaluate the capacity of microorganisms to perform biodegradation under *in situ* conditions, which is required in bioremediation studies.

This work aimed to evaluate the hydrocarbon degradation ability of four bacterial strains and four metagenomic clones, all derived from petroleum reservoirs, in artificial seawater microcosms using crude oil as carbon and energy source. These aims were based on the hypothesis that microorganisms derived from petroleum reservoirs and known to be hydrocarbon degraders would efficiently degrade crude oil, being good candidates for further application in a mesocosm scale.

2. Materials and methods

2.1. Microorganisms

The bacterial strains and metagenomic clones used in the present work were derived from Brazilian petroleum reservoirs (Campos Basin, RJ and Potiguar Basin, RN) and were previously evaluated for their ability to degrade different classes of hydrocarbons (Vasconcellos et al. 2009, 2010, 2011; Sierra-Garcia et al., 2014). The bacterial strains were deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI) (Table 1). The metagenomic clones consist of *Escherichia coli* host cells harboring a DNA vector (in this case, a fosmid) containing a large fragment (~40 kb) of environmental DNA (for details, see Vasconcellos et al., 2010).

2.2. Detection of catabolic genes

PCR assays were performed with the bacterial strains to detect the catabolic genes alkane monooxygenase (*alk*) and aromatic ring dioxygenase hydroxylases (ARDHs), involved in the aerobic degradation of alkanes and aromatic compounds, respectively, using two sets of degenerate primers, according to Kuhn et al. (2009) (Table 2).

PCR amplification of the two catabolic genes was performed independently using the respective primer sets (Table 2). Twenty-five microliter reaction mixtures contained 5 μl of total DNA, 2 U *Taq* DNA Polymerase (Invitrogen Life Technologies), 0.2 mM dNTP mix (GE Healthcare), 1.2 μM of each primer, 1X *Taq* buffer and 1.2 mM $MgCl_2$. PCR amplification was conducted using an Eppendorf Mastercycler Gradient (Eppendorf Scientific, New York, USA), and the amplification programs consisted of 1 cycle for denaturation at 97 °C for 3 min, 30 cycles at 94 °C for 1 min, 51 °C (*alk* gene) or 58 °C (ARHD gene) for 1 min and 72 °C for 1 min, and a final extension cycle at 72 °C for 5 min. DNA from *Rhodococcus erythropolis* DSM 43066 and *Pseudomonas putida* CBMAI 994 was used as positive control for the *alk* and ARHD gene, respectively. The amplification products were checked by electrophoresis in 1.2% (wt vol-1) agarose gels.

2.3. Microcosms and crude oil extraction

Oil spills were simulated in microcosms assays that were set up in Erlenmeyer flasks containing 40 mL of artificial seawater (Osterhage et al., 2000), 0.1% yeast extract and 1% petroleum as carbon source. Non-biodegraded petroleum from the PTS 2 well, Potiguar Basin (RN, Brazil), was used as the carbon source. A pre-inoculum was performed in nutrient broth (NB) for the bacterial strains and in Luria Broth (LB) containing chloramphenicol (12.5 μg/ml) for the fosmid clones. The inoculum was standardized to 10^6 cells/mL for all microorganisms, in accordance to Cerqueira et al. (2011). Microorganisms were inoculated individually and in consortia (only for the metagenomic clones). The assays were

Table 1
Microorganisms used in microcosms assays for the evaluation of petroleum degradation.

Microorganisms	Source
Clone 1A	Metagenomic fosmid library, Potiguar Basin, RN Vasconcellos et al. (2010)
Clone 2B	Metagenomic fosmid library, Potiguar Basin, RN Vasconcellos et al. (2010)
Clone 9E	Metagenomic fosmid library, Potiguar Basin, RN Vasconcellos et al. (2010)
Clone 10A	Metagenomic fosmid library, Potiguar Basin, RN Vasconcellos et al. (2010)
Micrococcus sp. CBMAI 636	Formation water, Campos Basin, RJ Vasconcellos et al. (2009)
Dietzia maris CBMAI 705	Oil, Campos Basin, RJ Vasconcellos et al. (2009)
Bacillus subtilis CBMAI 707	Oil, Campos Basin, RJ Vasconcellos et al. (2009)
Achromobacter xylosoxidans CBMAI 709	Formation water, Campos Basin, RJ Vasconcellos et al. (2009)

performed in triplicate and incubated in a rotational shaker at 120 rpm at 37 °C for the metagenomic clones and at 28 °C for the bacterial strains. The different cultivation conditions were adopted based on the different growth requirements of each microorganism. The microcosms were monitored for 21 days, being evaluated for petroleum degradation every seven days. Triplicate Erlenmeyer flasks were sacrificed at each time point (0, 7, 14 and 21 days), and oil extraction was carried out following the methodology previously described by da Cruz et al. (2008). The extracted oil was then separated into SARA components: saturated (eluted with hexane), aromatic (hexane: DCM, 8:2 v/v) and polar fractions (resins and asphaltenes) (DCM:MeOH 95:5 v/v) using silica gel column chromatography, as described in ASTM D2007. The percentages of the saturated and aromatic fractions were obtained by the weight of each fraction divided by the oil weight (wt%). The percentages for the resin and asphaltene fractions were obtained by subtracting the percentage of saturated and aromatic fractions from 100%.

2.4. Gas chromatography (GC)

The GC–FID (flame ionization detection) measurements of whole oil for all samples were carried out for n-alkanes (*n*-C15 to *n*-C32 range) and isoprenoids. A GC–FID instrument (6890 N, Agilent Technologies, USA) equipped with a HP-5 capillary column (30 m length, 0.32 mm i.d.; film thickness, 0.25 μm) with He as the carrier gas at a constant flow rate was used. The injector was set at 290 °C, and the GC oven temperature was programmed from 40 °C to 310 °C (19 min isothermal) at 6 °C/min. The flame ionization detector was at 310 °C. All samples were analyzed in duplicate at a concentration of 0.02 mg/μL of oil in dichloromethane (DCM).

Biomarker diagnostic parameters of biodegradation (Table 3) were calculated based on whole oil chromatogram analysis. Pr/n-C17 and Ph/n-C18 from respective areas of phytane, pristane, heptadecane and octadecane and CPI were calculated according to the equation: $IPC14\text{-}32 = [2 \times \Sigma\ (nC_{15}\ a\ nC_{31})]/[nC_{14} + 2 \times \Sigma\ (nC_{16}\ to\ nC_{30}) + nC_{32}]$.

2.5. Gas chromatography–mass spectrometry (GC–MS)

The saturated and aromatic hydrocarbon fractions from the SARA separation were analyzed using GC–MS to evaluate the distribution of the biomarker compounds. Analysis was performed using an Agilent 6890 N GC instrument connected to an Agilent 5973 MSD mass detector, equipped with a DB5-MS fused silica column (30 m length, 0.25 mm i.d.; film thickness, 0.25 μm). The

Table 2
Degenerate primers used in PCR reactions for the detection of catabolic genes.

Primer	Target gene	Sequence (5′ → 3′)	Direction	Expected amplicon size (bp)	Reference
AlkF	Alkane monooxygenase	GCI CAI GAR ITI RKI CAY AA	Forward	524	Kuhn et al. (2009)
AlkR	Alkane monooxygenase	GCI TGI TGI TCI SWR TGI CGY TG	Reverse		
ARDH F	α-Subunit of ARHD	TTY RYI TGY AII TAY CAY GGI TGG G	Forward	329	Bellicanta (2005)
ARDH R	α-Subunit of ARHD	AAI TKY TCI GCI GSI RMY TTC CA	Reverse		

Table 3
Biomarker diagnostic parameters from whole oil analysis by GC–FID.

Biomarker parameters[1]	Time (days)	Microcosms									
		Control sample	Clone 2B	Clone 10[a]	Clone 1A	Clone 9E	CBMAI 705[d]	CBMAI 636[e]	CBMAI 709[f]	CBMAI 707[g]	Clone pool
Pr/nC_{17}[a]	7	0.86	0.85	0.86	0.86	0.85	1.25	1.33	0.92	0.89	0.87
	14		0.87	0.86	0.86	0.90	1.75	1.40	0.92	0.87	0.87
	21		0.88	0.89	0.87	0.89	nd	nd	0.93	0.89	0.88
Ph/nC_{18}[b]	7	0.33	0.32	0.32	0.32	0.33	0.81	0.54	0.33	0.34	0.33
	14		0.33	0.32	0.33	0.31	0.86	0.92	0.33	0.33	0.32
	21		0.36	0.34	0.32	0.34	nd	nd	0.33	0.37	0.36
CPI[c]	7	1.06	1.06	1.06	1.06	1.06	1.06	1.07	1.06	1.06	1.06
	14		1.02	1.06	1.06	1.06	1.07	1.07	1.06	1.06	1.06
	21		1.06	1.06	1.06	1.06	nd	nd	1.06	1.06	1.06

[a,b] Calculated from the respective areas in the phytane (Ph), pristane, heptadecane and octadecane chromatogram from whole oil.
[c] Calculated from the respective areas in the *n*-alkanes chromatogram from whole oil, according to the equation: $CPI_{14\text{-}32} = [2 \times \Sigma\ (nC_{15}\ a\ nC_{31})]/[nC_{14} + 2 \times \Sigma\ (nC_{16}\ to\ nC_{30}) + nC_{32}]$.
[d] CBMAI 705 = *Dietzia maris*.
[e] CBMAI 636 = *Micrococcus* sp.
[f] CBMAI 709 = *Achromobacter xylosoxidans*.
[g] CBMAI 707 = *Bacillus subtilis*; *nd = not determined.
[1] Calculated in triplicate with standard deviations ranging from ±2,3 to ±5,8.

injector temperature for analysis of saturated hydrocarbons was set at 280 °C, and the oven temperature was increased from 60 °C (2 min isothermal) to 200 °C at 22 °C/min (3 min isothermal) to 300 °C at 3 °C/min (held 25 min). For the analysis of aromatic hydrocarbons, the injector temperature was set at 300 °C, and the oven temperature was increased from 70 °C (1 min isothermal) to 110 °C at 12 °C/min (22 min isothermal) to 320 °C at 1.5 °C/min (held for 3 min). He was the carrier gas at a constant flow of 1 mL/min. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV and 280 °C source temperature. Full scan mass spectra were recorded over a range of m/z 400–700, and Selected Monitoring Ions (SIM) analysis was carried out to m/z 85 for n-alkanes, m/z 191 for terpanes, m/z 178 for phenanthrene and m/z 192 for methylphenanthrenes (3-MP, 2-MP, 9-MP and 1-MP). Assignment of biomarker compounds was carried out by comparison with literature data (Peters et al., 2005). The saturated and aromatic hydrocarbon fractions were analyzed in duplicate at a concentration of 0.02 mg/μL of oil in hexane and DCM, respectively.

The biodegradation rates (%) for saturated (Fig. 3) and aromatic (Fig. 5) hydrocarbons were calculated using 17α(H), 21β(H)-hopane and chrysene as conservative markers (CM), respectively, applying the equation: Biodegradation rate (%) = $[1 - (A_{\text{hydrocarbon}}\ t/A_{\text{hydrocarbon}}\ t0) \times (A_{\text{CM}}\ t0/A_{\text{CM}}\ t)] \times 100$, where $A_{\text{hydrocarbon}}\ t$ and $A_{\text{hydrocarbon}}\ t0$ are the hydrocarbon relative peak areas at time t and $t0$ (control); $A_{\text{CM}}\ t0$ and $A_{\text{CM}}\ t$ are the conservative markers relative peak areas at time t and $t0$. In this work, the weathering effect of hydrocarbons was not evaluated in the natural weathering process (including evaporative loss, emulsification, dissolution, photo-degradation, etc.).

3. Results and discussion

3.1. SARA analysis

SARA fractionation separates the crude oil into four main classes based on polarity and solubility: (i) saturated hydrocarbons (n-alkanes, branched alkanes and cycloalkanes), (ii) mono and polyaromatic hydrocarbons, (iii) resins and (iv) asphaltenes. The concentration of these classes is commonly used for oil quality estimation utilized in upstream operations.

The oil SARA composition is generally closely related to oil viscosity. For example, a viscosity increase is often observed as asphaltene content increases and saturated hydrocarbon concentration decreases (Peralta-Martínez et al., 2011). Severely biodegraded oils are generally enriched in polar compounds and depleted in saturated hydrocarbon compounds. The results obtained from SARA composition analysis allowed for the evaluation of compound proportions and a qualitative estimate of which microorganisms are potentially degraders. In general, the SARA analysis revealed a higher percentage of NSO polar components (resins and asphaltenes, ranging from 26% to 45%) after 21 days than after 7 days for almost all samples, as shown in the ternary diagram (Fig. 1). Because the percentage of polar compounds increases with further biodegradation, these results suggest that all microorganisms shown in the diagram are potential degraders.

3.2. Whole oil analysis by GC–FID

The petroleum compounds revealed by GC–FID analysis represent one of the most employed parameters in monitoring saturated hydrocarbon degradation, as these are the first compounds to be consumed by microorganisms. Based on this analysis, light to moderate biodegradation of crude oils can be assessed by calculating the ratios of branched and cyclic alkanes over n-alkanes and by observing the typical "hump" or unresolved complex mixture (UCM) of branched and cyclic compounds that is characteristic of biodegraded oils (Peters and Moldowan, 1993).

Fig. 2 illustrates whole oil chromatograms of the control sample (t = 0 days), metagenomic clone 2B, bacterial strains *Dietzia maris* CBMAI 705 and *Micrococcus* sp. CBMAI 636 and the clone pool, which are the microorganisms that showed the greatest potential for biodegradation, at days 14 and 21 of the biodegradation experiment.

The chromatographic profiles of whole oil for the control sample (t = 0 days) revealed the presence of saturated hydrocarbons in the nC$_{14}$ to nC$_{36}$, suggesting a non-biodegraded sample (PM level 0), according to Peters and Moldowan (1993) scale, with uniform distribution of the even and odd homologues. However, the chromatographic profiles after 21 days demonstrated that biodegradation was low to moderate for all microorganisms assessed in this work (Fig. 2). The biodegradation rates (%) for n-alkanes (nC$_{14}$ to nC$_{33}$) and isoprenoids (Pr and Ph) for all investigated microorganisms are depicted in Fig. 3.

The metagenomic clones 1A and 2B, with biodegradation rates of 31% and 47%, respectively, after 21 days compared to the control sample, were the best performing microorganisms for saturated hydrocarbon degradation. Among the bacterial strains, *Dietzia maris* CBMAI 705 and *Micrococcus* sp. CBMAI 636 were more efficient in biodegrading n-alkanes and isoprenoids, with rates >99% after 21 days. The clone pool biodegraded these compounds at rates from 11 to 45% (Fig. 3).

Additionally, this work used the parameters pristane/n-heptadecane (Pr/n-C17) and phytane/n-octadecane (Ph/n-C18), which are most commonly used to describe the extent of microbial alteration in low to moderately biodegraded crude oils. In general, it is

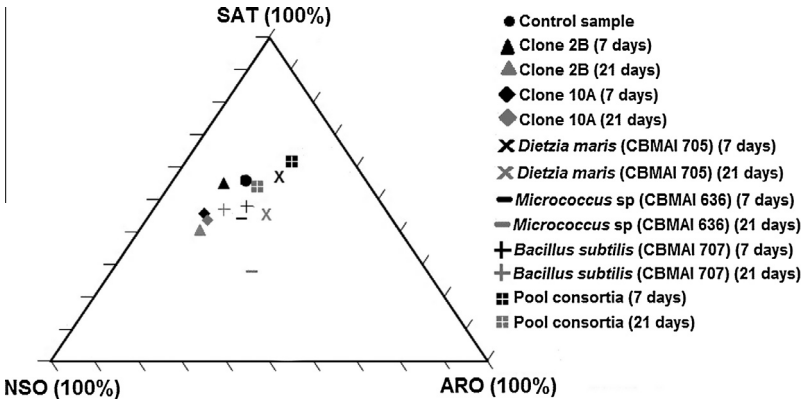

Fig. 1. Ternary diagram of SARA analysis for the evaluated microorganisms in the control (t = 0 days) and after 7 and 21 days of incubation. SAT = saturated hydrocarbon fraction; ARO = aromatic hydrocarbon fraction; and NSO = Polar components (resins and asphaltenes).

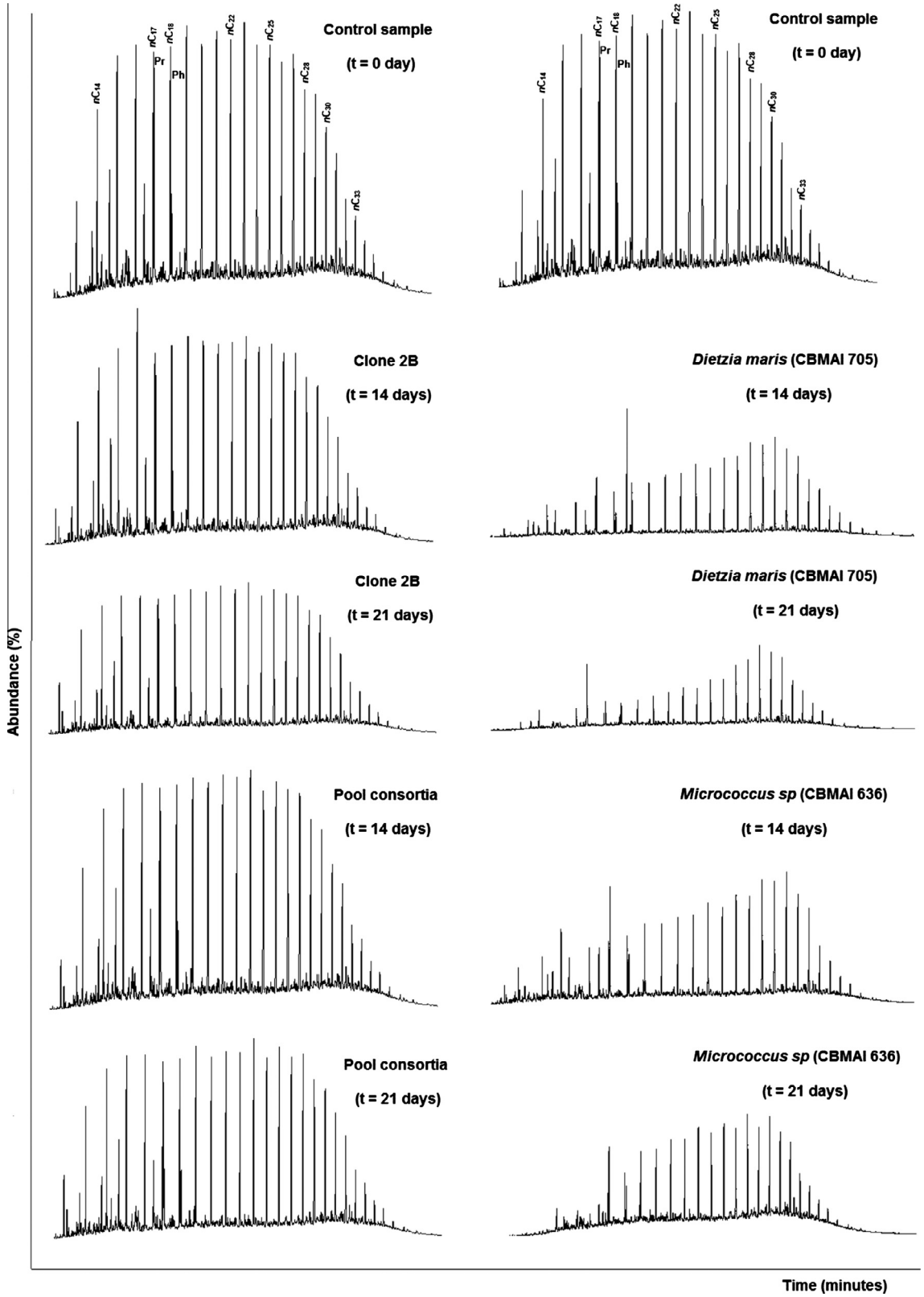

Fig. 2. Whole oil chromatograms from extracts of the control sample, metagenomic clone 2B, *Dietzia maris* CBMAI 705, *Micrococcus* sp. CBMAI 636 and the clone pool during the biodegradation experiment after 14 and 21 days (nC_{14} to nC_{33} = hydrocarbons; Pr = pristine; Ph = phytane) .

believed (Peters and Moldowan, 1993; Wenger and Isaksen, 2002) that pristane and phytane are not depleted by initial to moderate biodegradation, while n-alkanes are clearly affected. In moderately biodegraded samples (level 3, according to Peters and Moldowan scale – PM 3), these ratios are higher than in non-biodegraded oils or lightly biodegraded samples (PM 2). In contrast, in highly biodegraded oil (PM > 6), these ratios tend toward zero due to the absence of these compounds (nC_{17}, Pr, nC_{18} and Ph), and only an

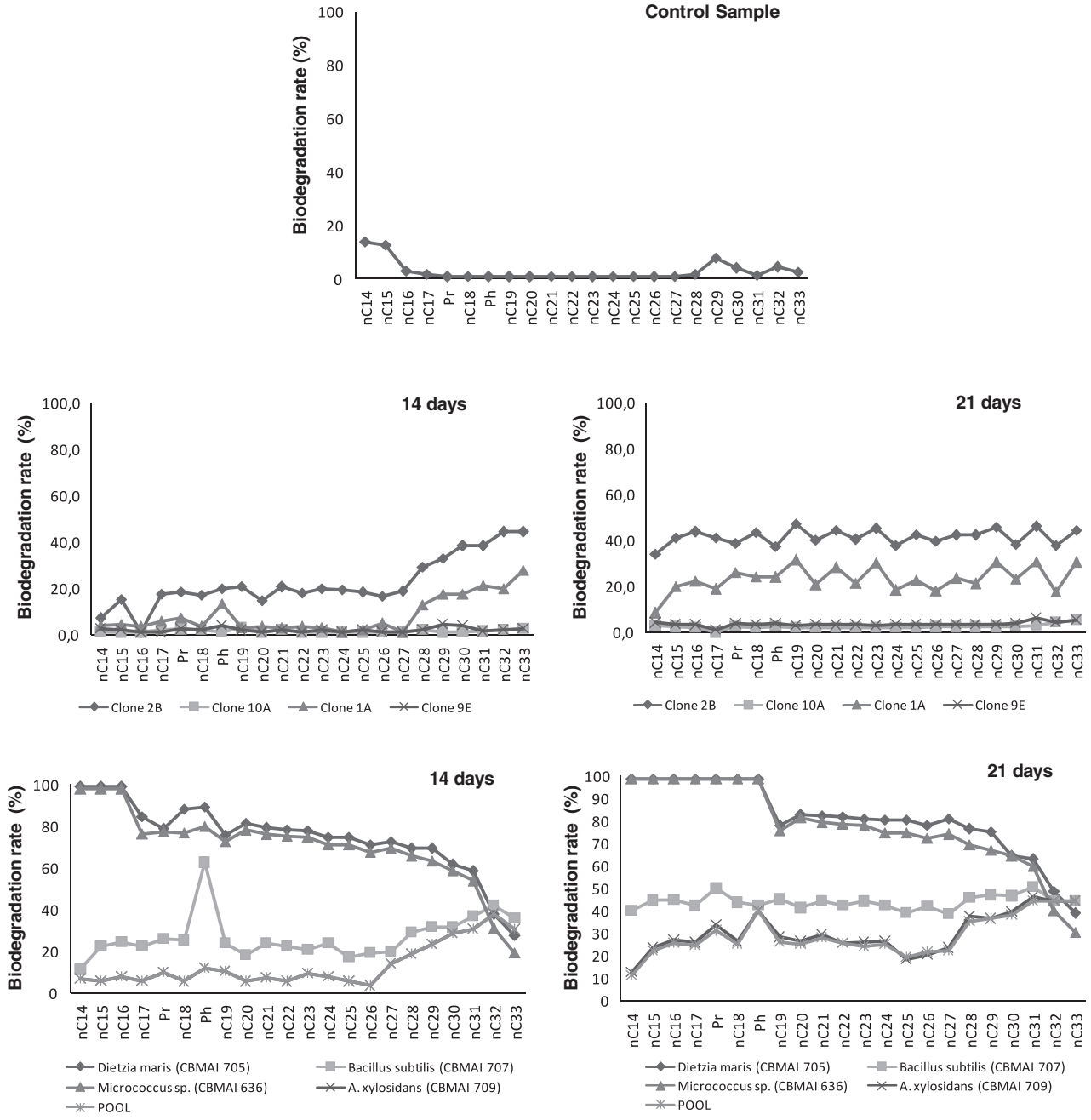

Fig. 3. Biodegradation rates (%) of the *n*-alkanes (nC_{14} to nC_{33}) and isoprenoids (Pr and Ph) from extracts of the control sample and all microcosms evaluated during the biodegradation experiment after 14 and 21 days.

elevation in the baseline (increased UCM) is observed. Consequently, increasing values of Pr/n-C17 and Ph/n-C18 indicate increasing biodegradation. In this work, in general, there was a slight increase in these ratios after 21 days compared to the control for most microorganisms evaluated (Table 3).

On the other hand, CPI analysis was not adequate to differentiate the biodegradation ability of the microorganisms because all showed CPI > 1. However, this result (CPI > 1) demonstrated that all microorganisms evaluated in this work prefer to degrade n-alkanes with odd carbon numbers compared to n-alkanes with even carbon numbers. This is contrary to the results reported in da Cruz and co-workers (2011), where microorganisms showed a preference for n-alkanes with an even number of carbons under anaerobic conditions. Therefore, this parameter (CPI) should be used with caution to monitor preferential biodegradation of n-alkanes with odd or even carbon numbers.

The results obtained in this study showed the occurrence of n-alkane biodegradation before isoprenoid biodegradation, resulting in an increase in Pr/nC_{17} and Ph/nC_{18} ratios. Higher values in these ratios were observed for the best performing microorganisms, including the strains *Dietzia maris* CBMAI 705 and *Micrococcus* sp. CBMAI 636 (Table 3).

3.3. Terpane and aromatic biomarker analysis

According to the polycyclic alkane biomarker analysis, the biodegradation process has not exceeded moderate alteration levels because all microorganisms evaluated showed low biodegradation

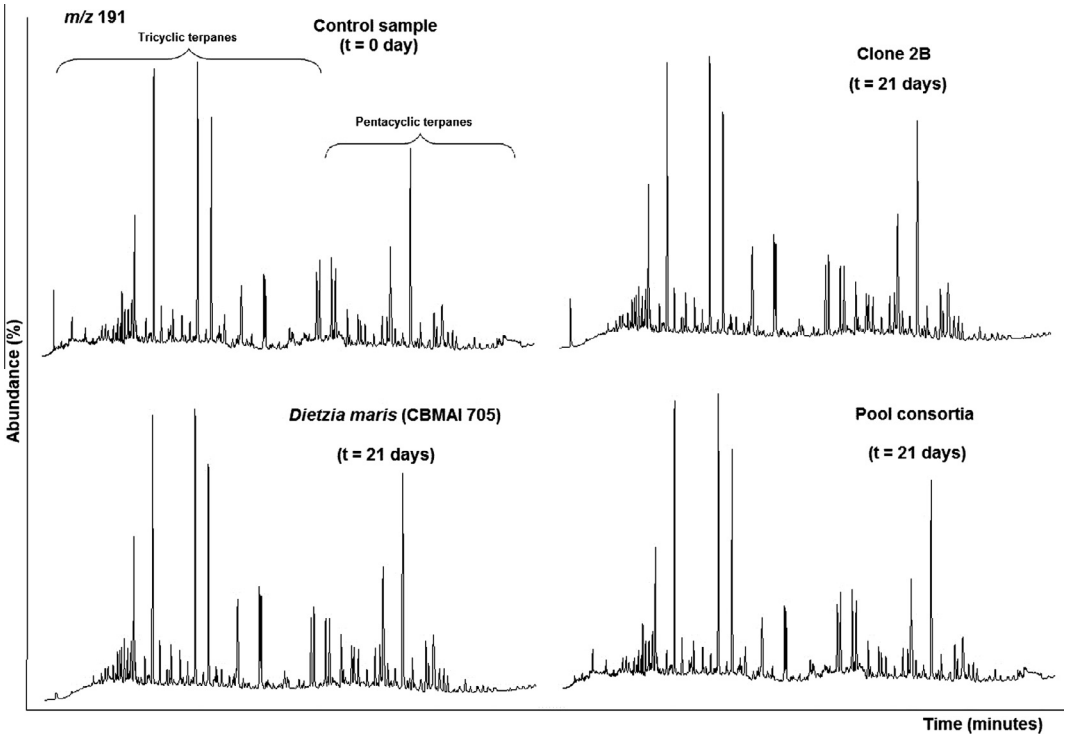

Fig. 4. GC–MS ion fragmentograms of terpanes (*m/z* 191) from extracts of the control sample, metagenomic clone 2B, *Dietzia maris* CBMAI 705 and the clone pool during the biodegradation experiment after 21 days.

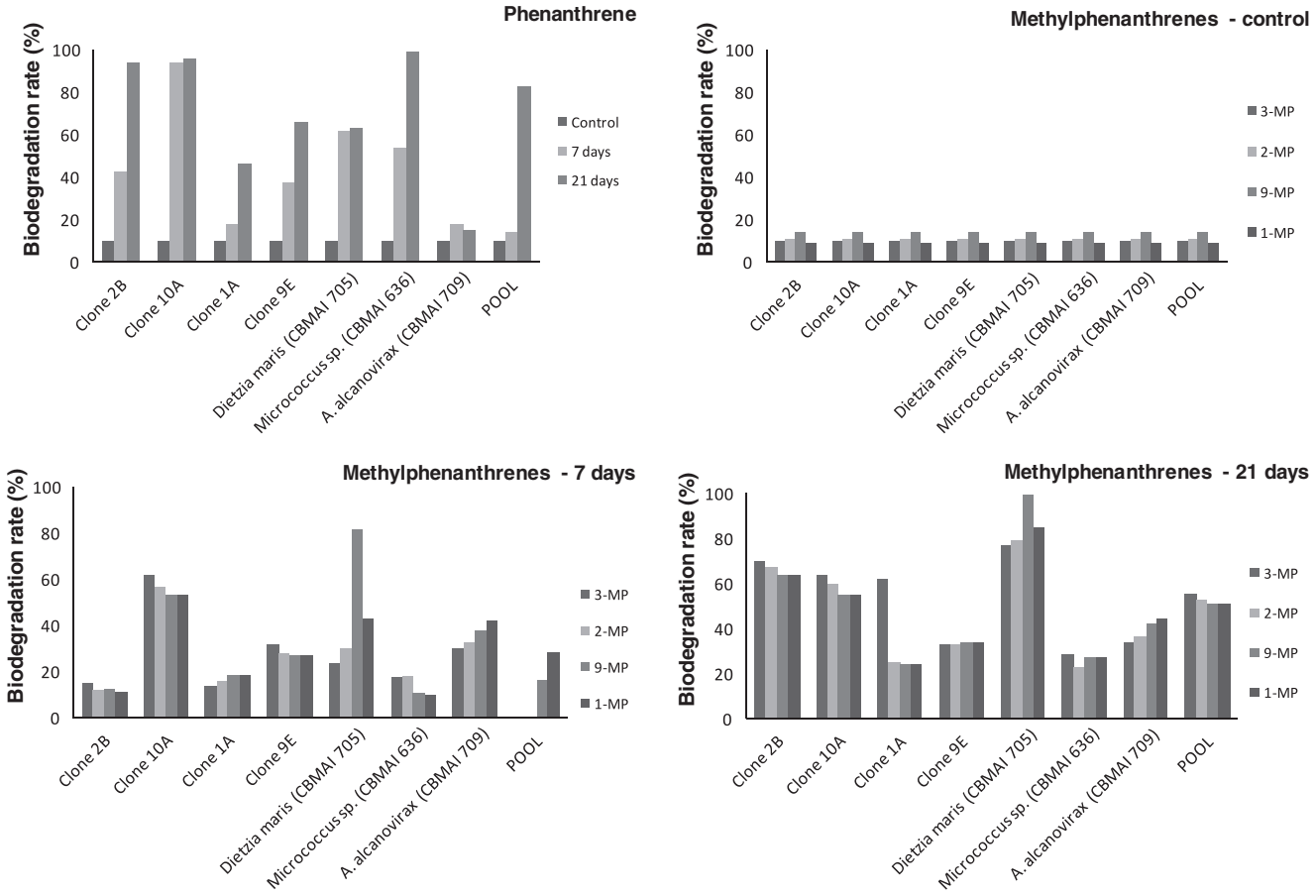

Fig. 5. Biodegradation rates (%) of the phenanthrene and methylphenanthrenes (3-MP, 2-MP, 9-MP, 1-MP) from extracts of the control sample and all microcosms evaluated during the biodegradation experiment after 7 and 21 days.

of terpanes (Fig. 4). The biomarkers tricyclic and pentacyclic terpanes are recalcitrant with regard to early to moderate biodegradation (Wenger and Isaksen, 2002; Peters et al., 2005). However, the distribution of these compounds in the crude oil under study was not affected by the microorganisms in the microcosm assays (Fig. 4).

The compounds phenanthrene (*m/z* 178) and methyphenanthrenes (*m/z* 192) were analyzed as well. Several studies showed that aromatic compounds can be degraded after saturated hydrocarbons and prior to or concomitantly with terpane biomarkers in reservoir or environmental conditions during oil spills (Wang and Fingas, 1997), reflecting differences in the rate of microbial catabolism under varying conditions (Peters et al., 2005). The biodegradation rates of phenanthrene and methylphenanthrenes for metagenomic clones, bacterial strains and the clone pool are depicted in Fig. 5.

During the biodegradation experiment, the most distinct change in the abundance of phenanthrene was observed for the metagenomic clones 2B and 10A which consumed more than 90% of this compound in 21 days. Among the bacterial strains, *Micrococcus* sp. CBMAI 636 was the most efficient in biodegrading phenanthrene with rates up to 99% compared to the control sample. Methylphenanthrenes also underwent a significant decrease in abundance during this experiment. Although not as significant as for phenanthrene, the clones 2B and 10A were also more efficient for methylphenanthrenes, biodegrading 70 and 64%, respectively, after 21 days compared to the control sample. On the other hand, *Dietzia maris* CBMAI 705 was more efficient in the degradation of methylphenanthrenes compared to phenanthrene, consuming more than 99% in 21 days. The clone pool biodegraded more than 80% of the phenanthrene and approximately 60% of the methylphenanthrenes in 21 days. These results showed that under the conditions used in this experiment, the fosmid clones 2B and 10A, the bacterial strains *Micrococcus* sp. CBMAI 636 and *Dietzia maris* CBMAI 705 and the clone pool exhibited a considerably high bioremediation potential in the biodegradation of phenanthrene and methylphenanthrenes (see Fig. 6).

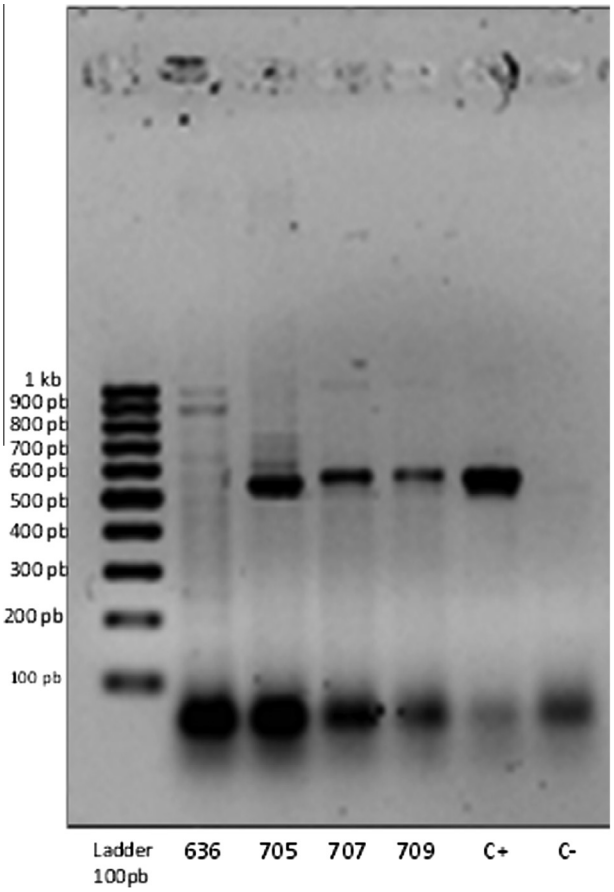

Fig. 6. Electrophoresis of PCR products in a 1.2% agarose gel obtained using primers targeting the *alk* catabolic gene and the bacterial strains. Left to right: 100 bp molecular weight marker (Fermentas), *Micrococcus* sp. CBMAI 636, *Dietzia maris* CBMAI 705, *Bacillus subtilis* CBMAI 707, *Achromobacter xylosoxidans* CBMAI 709, and positive and negative controls.

3.4. Microorganisms

In this work, the ability to degrade petroleum hydrocarbons was investigated in microcosms assays employing metagenomic clones and different bacterial isolates, all derived from petroleum reservoirs.

Among the metagenomic clones, the best performer was represented by clone 2B that displayed high performance in the ability to degrade saturated and aromatic hydrocarbons. Clone 10A showed high degradation rates for aromatic hydrocarbons, degrading 96% of the phenanthrene and 59% of the methyl phenanthrene after 21 days. Among the isolated strains, *Micrococcus* sp. CBMAI 636 and *Dietzia maris* CBMAI 705 were considered the best performers, particularly *Dietzia maris,* which was able to degrade both saturated and aromatic hydrocarbons.

The bacterial genera evaluated in this work have been mentioned previously in the literature as efficient hydrocarbon degraders. Although *Bacillus subtilis* CBMAI 707 presented an average performance in petroleum degradation in this study, this genus is often associated with petroleum degradation and biosurfactant production. PCR assays performed previously showed that *Bacillus subtilis* strain CBMAI 707 harbors the gene *srfA*, one of the genes belonging to a gene cluster involved in surfactin synthesis (Dellagnezze et al., 2010). Members belonging to the *Achromobacter* genus have also been previously isolated from petroleum-contaminated environments (Hassanshahian et al., 2013; Tambekar and Gadakh, 2012). However, in the present work, the best petroleum-degrading microorganisms were the strains belonging to the genera *Micrococcus* and *Dietzia,* which were already known as efficient hydrocarbon degraders (Ramsay et al., 2000; von der Weid et al., 2007; Bødtker et al., 2009; Jegan et al., 2010). *Dietzia* spp. was reported as a biosurfactant producer (Wang et al., 2013) and their potential application in the bioremediation technique has been investigated (Gharibzahedi et al., 2013). Although *Micrococcus* spp. are involved in some human infections, these bacteria are often described in the literature as being isolated from hydrocarbon-contaminated environments (Malik and Ahmed, 2012), producing biosurfactants (Palme et al., 2010; Tuleva et al., 2009) and being used in bioremediation studies (Silva-Castro et al., 2012).

Metagenomic libraries are a valuable tool often used in studies for the characterization of poorly understood environments, such as deep oceans (Quaiser et al., 2011; Dick et al., 2013), deserts (Neveu et al., 2011, forests (Faoro et al., 2011; Biver and Vandenbol, 2013), and petroleum reservoirs (Silva et al., 2013). The application of genetically modified organisms (GMOs) in contaminated environments has been described previously (Sayler et al., 1999; Ripp et al., 2000). However, the release of this type of microorganism in the environment and their commercialization still face a bottleneck, following examination by government regulatory agencies and risk assessments (Sayler and Ripp, 2000). Nevertheless, studies aimed at the application of fosmid clones carrying genes of biotechnological interest in bioremediation still are not described in the literature. The fosmid clones under study were previously shown to degrade hexadecane and phenanthrene (Vasconcellos et al., 2010; Sierra-Garcia et al., 2014). The authors

showed that fosmid clones have an excellent ability to degrade hexadecane (clones 2B, 1A and 10A) and a moderate ability to degrade phenanthrene (clones 10A and 2B). In addition, these studies unraveled the genetic organization of the metabolic pathways related to hydrocarbon degradation present in the fosmid clone inserts. The data obtained by these authors are in accordance with the results gathered in the present work, which demonstrated that clones 2B and 10A were able to degrade aromatic portions of petroleum after 21 days. It is worth mentioning that these fosmid clones were derived from a metagenomic library constructed using mixed community DNA obtained from aerobic and anaerobic bacterial enrichments. Therefore, the potential application of such clones becomes even more interesting, as they carry insert fragments with metabolic pathways from different degradation systems.

3.5. Detection of catabolic genes

PCR assays were carried out only with the bacterial strains and revealed that except for *Micrococcus* sp. CBMAI 636, all bacteria under study harbored the *alk* genes, which corroborated with a good alkane degradation performance, mainly by *Dietzia maris* CBMAI 705. The lack of amplification signal showed by *Micrococcus* sp. CBMAI 636 could possibly be explained by the low sequence similarity between the primers used and the *alk* sequence harbored by this strain (Fig. 6).

Works mentioning the presence of the *alk* genes in the *Dietzia* genus are described in the literature. Bihari et al. (2011) investigated the genetic background of long chain alkane degradation in strains belonging to *Dietzia* spp. In another study, the Dietzia strain DQ12-45-1b, isolated from the production water of a deep subterranean oil reservoir, was reported to harbor *alk*B (Wang et al., 2011).

Although almost all strains harbored the *alk* genes, the biodegradation process depends not only on the presence of the gene encoding the enzyme responsible for the hydrocarbon modification but also on environmental conditions, such as temperature, pH and nutrient availability. Thus, the different strains evaluated in this study have their own preference in relation to the carbon source, which could explain the variation in the alkane degradation observed among strains. Regarding the ARDH genes, no amplification signal was observed for the primers and conditions used in the PCR reactions. However, the metabolic pathways related to aromatic hydrocarbon degradation are diverse (Phale et al., 2007), and the strains in this study may harbor another group of genes related to aromatic degradation, showing low or no sequence similarity with the primers used.

Nonetheless, according to the degradation performance of saturated and aromatic hydrocarbons observed in this work under controlled laboratory conditions, these microorganisms could be promising candidates for future use in bioremediation strategies because, in addition to their ability to degrade hydrocarbons, these microorganisms were previously shown to produce biosurfactants (Vasconcellos et al., 2011).

4. Conclusions

In this work, the degradation potentials of different microorganisms, including bacterial isolates and metagenomic clones, derived from petroleum reservoirs were evaluated in microcosm assays using crude oil as a substrate. Chemical analyses demonstrated biodegradation rates higher than 99% for saturated hydrocarbons and aromatic compounds for some microorganisms assessed when compared to the control after only 21 days. The most efficient microorganisms for *n*-alkane and isoprenoid degradation were represented by the metagenomic clones 2B and 1A and the bacterial isolates *Dietzia maris* CBMAI 705 and *Micrococcus* sp. CBMAI 636. Furthermore, a considerably high biodegradation potential was confirmed in the biodegradation of phenanthrene and methylphenanthrenes, mainly for the metagenomic clones 2B and 10A and the bacterial strains *Dietzia maris* CBMAI 705 and *Micrococcus* sp. CBMAI 636. However, all microorganisms showed low efficiency for the degradation of terpanes. Finally, it is worth highlighting the outstanding performance of clones 2B, 1A and 10A for crude oil degradation, considering that in this case, the degradation genes are working in a heterologous host. This work reports innovative results for hydrocarbon degradation by using genetic resources from petroleum reservoirs, opening perspectives for their future use in bioremediation strategies in oil impacted areas.

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