Culturable populations of Acinetobacter can promptly respond to contamination by alkanes in mangrove sediments

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ABSTRACT

This study evaluated the potential of bacterial isolates from mangrove sediments to degrade hexadecane, an paraffin hydrocarbon that is a large constituent of diesel and automobile lubricants. From a total of 18 oil-degrading isolates obtained by an enrichment technique, four isolates showed a great potential to degrade hexadecane. The strain MSIC01, which was identified by 16S rRNA gene sequencing as Acinetobacter sp., showed the best performance in degrading this hydrocarbon, being capable of completely degrading 1% (v/v) hexadecane within 48 h without releasing biosurfactants. Its hydrophobic surface probably justifies its potential to degrade high concentrations of hexadecane. Thus, the sediments from the studied mangrove harbour bacterial communities that are able to use oil as a carbon source, which is a particularly interesting feature due to the risk of oil spills in coastal areas. Moreover, Acinetobacter sp. MSIC01 emerged as a promising candidate for applications in bioremediation of contaminated mangrove sediments.

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1. Introduction

Mangroves are coastal ecosystems that occur in tropical and subtropical regions of the world, occupying intertidal areas. These ecosystems are particularly susceptible to oil contamination since they are usually located next to active regions of oil production, transportation and other anthropogenic activities, acting as efficient reservoirs of pollutants derived from the sea, rivers, groundwater and continent (Burns et al., 1993). The intrinsic characteristics of these environments, including the relatively high content of sulfide and organic matter, anoxic sediments and reduced stream flow, may facilitate the deposition and accumulation of contaminants (Ke et al., 2005).

During recent decades, the extensive use of oil has increased the risk of releasing toxic hydrocarbons into the environment, especially in mangroves, which are highly vulnerable to pollution (Burns et al., 1993). Among the mechanisms used to control the fate of the oil after contamination, bioremediation – a cleaning process that uses living organisms such as microorganisms or their metabolites – offers new opportunities for treating polluted sediments. In addition, bioremediation has a low cost as well as the possibility of a complete mineralization of pollutants, eliminating the risk of further contamination and increased level of public acceptance (Santos et al., 2010).

It is well known that oil-degrading bacteria occur extensively in a wide variety of environments, including sediments and the water column (Ramsay et al., 2000). The use of native or indigenous microflora for bioremediation is of great interest since it is often more useful and beneficial than commercial inoculants (Grosser et al., 1995). Moreover, due to existing restrictions on the introduction of exogenous microorganisms into the environment, it is necessary to determine the biodegradation potential of native species and understand the biotransformation of pollutants by microbial communities that are already adapted to a particular environment (MacCormack and Fraile, 1997).

When close to human activities, mangroves show very high values of organic matter and accumulate large amounts of anthropogenic pollutants. Thus, it is not surprising that mangrove microorganisms have developed the ability to metabolize organic compounds such as oil hydrocarbons (Atlas, 1981).

Aliphatic hydrocarbons are the main constituents of crude oil, especially alkanes (C14–C20), making biodegradation quantitatively the most important process in removing crude oil from the environment (Head et al., 2006). Despite the greater attention received by aromatic contaminants, they represent less than 5% of the total volume of oil (Stroud et al., 2007).

Studies investigating the fate of aliphatic hydrocarbons in sediments are extremely important. Due to the lack of functional...
groups and low solubility in water, the aliphatic hydrocarbons exhibit low chemical reactivity and bioavailability for microorganisms (Stroud et al., 2008). Loser et al. (1999) found that hexadecane remained in remediated soils and this persistence was linked to the limited bioaccessibility of the contaminant. However, some bacteria and fungi have metabolic potential to use these compounds as carbon and energy sources (Berthe-Corti and Fetzner, 2002).

In this context, this work evaluated the potential of indigenous bacteria to degrade petroleum hydrocarbons in mangrove sediments located in northeast Brazil in a region known for petroleum exploitation and considered as a risk area for oil contamination, aiming to provide information on the recovery of impacted mangrove areas.

2. Materials and methods

2.1. Study area and sediment sampling

Barra Grande mangrove is located in Icapuí, on the extreme east coast of the State of Ceará, northeastern Brazil (S 4°40’ W 37°20’), in a region comprised of an extensive tidal flat, covering an area of 1260.31 hectares (Fig. 1). Sediment was sampled during spring tide (0.1 m). Sediments from three different habitats at depths of 0–10 cm were collected following the shoreline in a perpendicular transect. Site 1 (S1) was the closest to the sea in an area without vegetation, the second site (S2), located in an area next to Avicennia shaueriana, and the third site (S3) in a region surrounded by a robust forest of Rhizophora mangle. The sites were 150 m apart from each other. At each habitat, five samples of sediments were removed from a 1 m² area, using a core with a 10 cm diameter and transferred to sterile flasks. Samples were taken to the laboratory on ice in order to keep the temperature around 4–8 °C. The five replicates of each site were homogenized and 25 g of the composite samples were immediately used for isolating microorganisms. Sediment granulometry was performed by dry sieving (Sugioio, 1973), and the organic matter was determined by weight loss on ignition, as described in Schulte and Hopkins (1996).

2.2. Enrichment culture and isolation of bacteria

Enrichment was conducted in Erlenmeyer flasks containing 225 mL of Bushnell and Haas – BH (1941) mineral medium containing 2% (w/v) NaCl (Salt BH [SBH]). The medium was autoclaved at 121 °C for 15 min and, after cooling, a 0.1% (v/v) micronutrient solution containing 10.95 g L⁻¹ ZnSO₄·7H₂O; 5.00 g L⁻¹ FeSO₄·7H₂O; 1.54 g L⁻¹ MnSO₄·H₂O; 0.39 g L⁻¹ CuSO₄·5H₂O; 0.25 g L⁻¹ Co (NO₃)₂·6H₂O; and 0.17 g L⁻¹ Na₂B₄O₇·10H₂O was aseptically added. The micronutrient solution was previously sterilized using a 0.22-μm membrane filter (Millipore, USA).

Twenty-five grams of the composite samples from each site were inoculated in flasks containing sterilized SBH medium. Oil from the extraction plant near the mangrove area (Fazenda Belém, state of Ceará, Brazil) was sterilized at 121 °C for 30 min and 1% (w/v) was added to each flask as the carbon source. Biweekly, subcultures were prepared by transferring 10 mL to 90 mL of fresh SBH. The cultures were maintained at 160 rpm at 32 °C for 60 days and, at the end of each incubation period, enriched cultures were grown in PCA (Plate Count Agar, Difco, USA) containing 2% (w/v) NaCl. After 48 h of incubation, the plates were analysed and

Fig. 1. Sampling sites (S1, S2 and S3) in Barra Grande mangrove, State of Ceará, Brazil.
distinct colonies were selected. The isolates were stored at –80 °C in Nutrient Broth (Merck, Germany) containing 15% glycerol (v/v).

2.3. Selection of hexadecane-degrading bacteria

In order to select the best hexadecane-degrading bacteria, 1 mL of cultures of the isolates containing 10⁷–10⁸ colony-forming units (CFU) were transferred to 9 mL of SBM medium supplemented with 1% (v/v) hexadecane as the carbon source (Kim et al., 2002). The SBM medium and hexadecane solution were sterilized by autoclaving at 121 °C for 15 min and filtration with a 0.22-µm membrane filter, respectively. The cultures were maintained at 160 rpm and 32 °C and aliquots were removed immediately after inoculation, and at 48 and 96 h for biomass analysis estimated by optical density at 600 nm. The analysis was run in triplicate with at least two repetitions. Simultaneously, controls using the same methodology, but without the addition of bacterial inoculum, were also run.

2.4. Biodegradation testing

Biodegradation assays were conducted in 250-mL Erlenmeyer flasks with 50 mL of SBH sterilized at 121 °C for 15 min. Hexadecane, previously sterilized with a 0.22-µm membrane filter, was added to the flasks at a concentration of 10% (v/v). The inocula were prepared as described in item 2.3. One mL from each previously selected culture was added to 50 mL of SBH. The cultures were maintained at 160 rpm, 32 °C and aliquots were removed immediately after inoculation, and at 24, 48, 72 and 96 h, for biomass analyses estimated by optical density at 600 nm. The strain that showed the best performance was used in further experiments in 1, 10, 15 and 20% (v/v) hexadecane. The biodegradation test was prepared as described above and the cultures were monitored at 24, 48, 72 and 96 h by pH, optical density at 600 nm and residual hexadecane quantification by gas chromatography. Concomitantly, controls using the same methodology without the addition of bacterial inoculum were run and analysed.

Production of surface-active compounds by the selected isolate was investigated by the oil–water mixture emulsification test, described by Iqbal et al. (1995). Briefly, 2.0 mL of cell-free culture supernatant was combined with the same volume of kerosene in a 10-mL tube, the tubes were vortexed for 2 min and left to stand for 15 min. The hydrophobicity of the selected isolate was evaluated by sequencing the 16S rRNA gene. DNA extraction was performed using a CTAB (cetyltrimethyl ammonium bromide)-based protocol (Warner, 1996). The yield was estimated by measuring the absorbance at 260 nm in a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA).

The 16S rRNA gene was amplified by PCR using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTATCCAGCAG-3') as described by Lane (1991). Reactions were carried out in a final volume of 25 µL containing 100 ng of genomic DNA (template), 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer and 1.0 unit of Taq DNA Polymerase (MBI Fermentas Inc., USA). PCR amplifications were performed in a thermocycler programmed for an initial denaturation step (95 °C for 2 min) followed by 34 cycles of 95 °C for 1 min, 50 °C for 15 s, and 72 °C for 1 min. After the last cycle, the reactions were further incubated at 72 °C for 10 min. PCR products were purified using the UltraClean PCR Clean-up Kit (MO BIO, USA), following the manufacturer’s instructions. The complete sequence of the purified amplicon was determined using the DYEanemic ET terminators cycle sequencing kit (Amersham Biosciences, USA), according to the manufacturer’s protocol. Both strands were sequenced using the primers 27F, 1525R, 782R (5'-AAGGAGGTATCCAGCAG-3') and 1100R (5'-AGGCTTGGCGCTGTWG-3'). Sequencing reactions were analysed in a MegaBACE 1000 DNA sequencer (Amersham Biosciences, USA). The near full-length 16S rRNA gene sequence was assembled using Phrap (Ewing and Green, 1998). The sequence obtained was then compared with those already deposited in public databases (EMBL, GenBank, DDBJ and PDB) using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

3. Results and discussion

3.1. Identification of alkene-degrading bacteria

Twenty bacterial isolates from Barra Grande mangrove sediments were obtained from enrichment using local oil. The bacterial strains were named MSIC01 to MSIC20; however, after four successive cultivations on PCA containing 2% (w/v) NaCl to ascertain the purity of the cultures, two isolates, MSIC03 and MSIC18, did not survive. Therefore, the work was performed using 18 isolates. Despite not having a history of contamination, this mangrove sediment shelters bacterial populations that are able to survive and increasing the temperature to 230 °C with a heating rate of 25 °C min⁻¹. Tests were run in triplicate.

2.6. Cell-surface hydrophobicity

The hydrophobicity of the selected isolate was evaluated according to the methodology known as MATH (Microbial Adhesion to Hydrocarbons), described by Kim et al. (2002). The isolate was grown in Nutrient Broth (Difco) with 2% (w/v) NaCl for 24 h at 160 rpm, 32 °C. Afterwards, aliquots of 3.0 mL of cell suspension, adjusted to an absorbance of 0.1 at 600 nm, equivalent to a cell density of 10⁷ CFU mL⁻¹, were transferred to tubes containing 0.1, 0.2, 0.3, 0.6 and 1.0 mL of hexadecane. After incubation at 32 °C for 10 min, the tubes were vortexed for 2 min and left to stand for 15 min. The aqueous phase was carefully recovered to measure the optical density at 600 nm. The percentage of hydrophobicity (H) was obtained from the equation: $H = 100\% \times \frac{1}{(DO_{600nm\ after\ mixing}/DO_{600nm\ before\ mixing})}$.
use this compound as a source of carbon and energy. This fact reinforces the idea that indigenous bacterial communities from uncontaminated mangroves have considerable potential to degrade hydrocarbons (Ramsay et al., 2000; Tam et al., 2002; Guo et al., 2005; Yu et al., 2005; Brito et al., 2006).

Some studies have shown that there is no significant correlation between hydrocarbon concentration in mangrove sediments and the ability of native microorganisms to degrade this compound. Indeed, physical and chemical properties of sediments from each mangrove and their indigenous microbial community structure, especially the types and quantities of microorganisms, are crucial for the ability to degrade hydrocarbons (Tam et al., 2002; Guo et al., 2005).

The isolates were tested for their ability to grow in 1% (v/v) hexadecane and three distinct groups were observed: a group that was able to grow efficiently (OD$_{600nm}$ between 1 and 1.6), composed of isolates named MSIC01, MSIC06, MSIC17 and MSIC20; a group that grew moderately (OD$_{600nm}$ between 0.2 and 0.4), including the isolates MSIC10, MSIC12, MSIC14 and MSIC16; and a third group of 10 isolates that were not able to metabolize 1% (v/v) of hexadecane.

The isolates that had the greatest potential to degrade hexadecane were mainly from site S3 (except for MSIC17, which was isolated from site S2), a region characterized by dense vegetation, rich in organic matter and with higher silt–clay contents (Table 1). Chaerun et al. (2004) reported a strong positive correlation between organic matter content and abundance of oil-degrading bacteria through five years of monitoring carbon concentrations and microbial activity.

### 3.2. Degradation of hexadecane by the selected strains

The isolates that showed the best growth rates in 1% (v/v) hexadecane were further evaluated to degrade increased hexadecane concentrations. The potential of the isolates MSIC01, MSIC06, MSIC17 and MSIC20 to metabolize 10% (v/v) hexadecane is shown in Fig. 2. MSIC01 was the only strain that was able to grow in that concentration. Therefore, it was chosen for further tests in higher concentrations of hexadecane (Fig. 3). The data showed that this strain was able to remove up to 30% of a 20% (v/v) hexadecane concentration.

Previous studies on mangroves have determined that hydrocarbon degradation is mediated by a wide variety of bacteria (Ramsay et al., 2000; Tam et al., 2002; Guo et al., 2005; Yu et al., 2005; Brito et al., 2006). Among the eighteen strains isolated from Barra Grande mangrove, four were selected for their potential to metabolize hexadecane. However, the possibility that the other strains have the potential to degrade other types of hydrocarbons cannot be ruled out.

The partial 16S rRNA gene sequence of MSIC01 was determined and it had a length of 1380 bp (GenBank accession number JF876296). Similarity searches on public nucleotide databases revealed that this sequence was 99% identical with the 16S rRNA gene sequence from Acinetobacter sp. J42 (EU143354). Therefore, the strain MSIC01 was classified as *Acinetobacter* sp. MSIC01.

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand (%)</td>
<td>92.0 ± 0.2</td>
<td>93.0 ± 0.15</td>
<td>67.3 ± 0.2</td>
</tr>
<tr>
<td>Silt + clay (%)</td>
<td>8.0 ± 0.2</td>
<td>7.0 ± 0.15</td>
<td>32.7 ± 0.2</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>2.4 ± 0.34</td>
<td>2.7 ± 0.25</td>
<td>8.4 ± 0.6</td>
</tr>
</tbody>
</table>

Organic matter (%) in Barra Grande mangrove sediments, State of Ceará, northeastern Brazil.

**Fig. 2.** Growth curves of the isolates MSIC01, MSIC6, MSIC17 and MSIC20 in mineral medium containing 2% (w/v) NaCl and 10% (v/v) hexadecane as a carbon source.

**Fig. 3.** Growth curves of the isolate MSIC01 in mineral medium containing 2% (w/v) NaCl and 10% (v/v) hexadecane as a carbon source at different concentrations (% v/v).

*Acinetobacter* spp. are strictly aerobic and widely distributed in the environment, such as in soil, water and living organisms. Members of this genus are able to grow in a wide variety of carbon sources (Abdel-El-Haleem, 2003; Barbe et al., 2004) and are known as good hydrocarbon degraders, including alkanes (Rosenberg et al., 1982; Noordman et al., 2002; Yu et al., 2005; Throne-Holst et al., 2006; Wentzel et al., 2007).

Gas chromatography showed that the strain MSIC01 was able to degrade 95% of an initial concentration of 1% (v/v) hexadecane within 48 h, and 40% of 10% (v/v) hexadecane in the same time. In higher concentrations, 15% and 20% (v/v) of hexadecane, 30% of the initial concentration was degraded. Many studies on biodegradation of hexadecane have used concentrations up to 15 g L$^{-1}$, which corresponds to 2% (v/v) (Olivera et al., 1997; Sckelsky and Shreve, 1999; Noordman et al., 2002; Chénier et al., 2003; Pepi et al., 2005). Therefore, MSIC01 was able to grow in concentrations 10 times higher.

Studies conducted with *Pseudomonas aeruginosa* in the presence of surfactants revealed that 60% of 0.9 g L$^{-1}$ hexadecane was consumed after 40 h (Sckelsky and Shreve, 1999). Olivera et al. (1997) used a bacterial consortium to obtain 95% degradation of 0.0928 g L$^{-1}$ of hexadecane after 120 h. Koma et al. (2001) tested the ability of a strain of *Acinetobacter* sp. to degrade a long-chain n-paraffin and found that the strain mineralized 0.1% (w/v) of this compound within 96 h. Gomes-Barreto et al. (2010) working with *Bacillus subtilis* LAM008 obtained similar results to this study, since that strain was able to degrade almost 100% of 1% (v/v) hexadecane within 48 h. Based on the studies cited above and the results obtained in this study, it can be stated that *Acinetobacter* sp. MSIC01 possesses a great ability to degrade hexadecane.
Several alkane oxidation routes have been described for Acinetobacter species. Biochemical data suggest that the cytochrome P-450 is a terminal hydroxylase in some strains that grow in long and average alkane chains. The involvement of dioxygenases in the degradation of long alkane chains has also been reported in Acinetobacter sp. M-1. Moreover, studies have shown that there is an alkane terminal hydroxylase dependent on rubredoxine and a rubredoxine reductase involved in the oxidation of long alkane chains in A. calcoaceticus 69-V and Acinetobacter sp. ADP1. Thus, alkane degradation routes differ in different strains of Acinetobacter (Maeng et al., 1996; Ratajczak et al., 1998). In this study the products of biodegradation were not identified, so it is not possible to state the route used by Acinetobacter sp. MSIC01.

Several strategies to overcome the low solubility of n-alkanes and to facilitate their transport through the membrane are used by bacteria. The hydrophobic nature of the bacterial cell surface plays an important role, as the contact with hydrophobic substrates is crucial for the initial stage of aliphatic and aromatic hydrocarbon degradation mediated by the oxygenases associated with the cell surface. In the case of long n-alkane chains, there are two capture mechanisms. The first involves hydrocarbon interfacial access by direct contact with the cell, mediated by cell-surface hydrophobicity, and the second is mediated by biosurfactants, which facilitate the contact of the cells with these compounds (Bouchez-Naitali et al., 2001; Kim et al., 2002; Wentzel et al., 2007; Cameotra and Singh, 2009).

In order to identify how MSIC01 captures hexadecane, the cell-surface hydrophobicity and the ability to emulsify kerosene were evaluated. MSIC01 showed high hydrophobicity (around 90%), measured after the contact with 0.3 mL of hexadecane, but it did not produce emulsification. Bouchez-Naitali et al. (2001) have demonstrated that several hydrophobic strains do not produce surfactants. Kim et al. (2002) stated that the transportation and selective accumulation of alkane occurs in Rhodococcus erythropolis S + 14He, a highly hydrophobic strain (80%). Gomes-Barreto et al. (2010) showed that vegetative cells and spores of B. subtilis LAM08 exhibited low hydrophobicity (maximum 25% when in contact with 1.0 mL of hexadecane). However, they detected production of biosurfactants, which was not observed in our study.

The data suggest that MSIC01 probably captured hexadecane through direct contact with the cell surface without releasing biosurfactants. It is known, however, that different species of Acinetobacter produce complex surfactants such as emulsan (A. calcoaceticus RAG-1) and alasan glycoproteins (A. radioresistens KA-53) (Desai and Banat, 1997).

In Acinetobacter sp. RAG-1, fimbriae are considered crucial for the growth of this strain in hexadecane and are responsible for its adherence to hydrophobic surfaces, making it able to capture and metabolize hydrocarbons (Rosenberg et al., 1982). Previous studies have shown that Acinetobacter species form intracellular inclusions of oil when growing in hexadecane in the absence of soluble carbon sources, suggesting that these structures are related to the hydrocarbon oxidation (Scott and Finnerty, 1976; Kim et al., 2002).

It is possible that MSIC01 has such structures and mechanisms that facilitate the hydrocarbon transportation since it did not produce biosurfactants, however it showed a great ability to use hexadecane as the sole source of carbon and energy, which strengthens the idea that the cell-surface hydrophobicity allows an efficient uptake of hydrophobic substances such as hydrocarbons.

4. Conclusions

The main contribution of this study was to show that pristine mangrove sediments, especially those rich in silt–clay and organic matter, harbour bacterial populations capable of degrading crude oil and derivatives, demonstrating that native microbiota can promptly respond to oil spills. Considering the potential to degrade hexadecane, Acinetobacter sp. MSIC01 isolated in this study, stands out as a promising candidate for applications in bioremediation of contaminated mangrove sediments.

Acknowledgements

The authors gratefully acknowledge FINEP/CT-Petrobras for financial support of the RECUPETRO (Re Rede Cooperativa em Recuperação de Áreas Contaminadas por Atividades Petrolíferas). L.L.R thanks CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for her doctoral scholarship.

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