Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities

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Abstract

Fifteen bacterial strains isolated from solid waste oil samples were selected due to their capacity of growing in the presence of hydrocarbons. The isolates were identified by PCR of the 16S rDNA gene using fD1 and rD1 primers. The majority of the strains belonged to genera Bacillus, Bacillus pumilus (eight strains) and Bacillus subtilis (two strains). Besides, three strains were identified as Micrococcus luteus, one as Alcaligenes faecalis and one strain as Enterobacter sp. Growth of the above-mentioned strains in mineral liquid media amended with naphthalene, phenanthrene, fluoranthene or pyrene as sole carbon source was studied and our results showed that these strains can tolerate and remove different polycyclic aromatic hydrocarbons that may be toxic in the environment polluted with hydrocarbons. Finally, the capacity of certain strains to emulsify octane, xylene, toluene, mineral oil and crude oil, and its ability to remove hydrocarbons, look promising for its application in bioremediation technologies.

Keywords: Bacillus; Alcaligenes; Micrococcus; Enterobacter; Hydrocarbon; PAH; Bioemulsifier

Introduction

Bioremediation of contaminated aquatic and soil environments has arisen as an effective technology, with a range of advantages compared to more traditional methods. Bioremediation of waste materials, which contain hydrocarbons and their derivatives, is based on the ability of microorganisms to increase their biomass growing on these substrates and degrading them to non-toxic products, such as H₂O and CO₂ [16].

Polycyclic aromatic hydrocarbons (PAHs) extensively occur as pollutants in soil and water, and are important environmental contaminants because of their recalcitrance. These compounds also mean a potential risk to human health, as many of them are carcinogens [11]. Their persistence within the ecosystems is due to their low aqueous solubility and high sorption to soil, two features that limit their availability for the degrading microorganisms. Bioemulsifiers have been also reported as enhancers of hydrocarbon biodegradation in liquid media, soil slurries and soil microcosms [20]. For instance, the dispersion of octadecane in aqueous solution is dramatically enhanced by application of 300 mg l⁻¹ of rhamnolipid biosurfactant [31].

Petroleum components have traditionally been divided into four fractions: saturated hydrocarbons, aromatic hydrocarbons, nitrogen–sulphur–oxygen containing compounds (NSO) and asphaltthenes. The relative proportions of these fractions vary from crude
to crude, and the susceptibility of a specific crude to microbial degradation can be predicted from its composition. Normally, the fractions contain n-alkanes and are mostly susceptible to biodegradation, whereas saturated fractions containing branched alkanes are less vulnerable to microbial attack. The aromatic fractions are even less easily biodegraded, and the susceptibility decreases as the number of aromatic or alicyclic rings in the molecule increases [5].

Microbial degradation of PAHs is considered to be the major decomposition process for these contaminants in nature, and represents a potential solution to the environmental problems posed by them [27]. It is difficult to elucidate the mechanisms that govern the biodegradation of PAHs in complex media containing mixtures of contaminant substrates and heterogeneous microbial communities. To study the composition of the natural populations contributing to the biotransformation and biodegradation processes in these complex systems, culture-independent approaches have been successfully applied [22]. However, bacteria naturally inhabiting contaminated sites are of interest as potential agents for PAHs bioremediation, and several papers have been focused on the isolation and characterization of strains with the ability to grow using PAHs, such as naphthalene, phenanthrene, fluoranthene and pyrene, as sole carbon and energy sources [1,4,7,32]. The degrading strains that have been characterized so far in the literature are taxonomically diverse, and mainly belong to the genera *Pseudomonas*, *Alcaligenes*, *Sphingomonas*, *Bacillus* and *Mycobacterium* [1,3,4,9].

In the present study, we describe the characterization of microbial strains with capacities to remove naphthalene, phenanthrene, fluoranthene or pyrene, and also to produce biosurfactants. The growth characteristics and emulsifying activity of these bacterial strains, as well as their identification by analysis of the sequence of the gene encoding 16S rDNA, are reported.

**Materials and methods**

**Microorganisms**

Microorganisms used in this study were part of a collection of 66 strains isolated from solid waste crude oil samples collected from the clean up of oil storage containers, which have been previously described [6]. The strains were selected for further studies due to their ability to grow on solid media supplemented with PAHs [6]. The collection of strains was long-term maintained in the laboratory by lyophilization. For this study, stock cultures were grown on TSA (Difco) slants.

**Culture media**

Growth of selected strains on PAHs was determined in BH liquid medium with the following composition (g l\(^{-1}\)): MgSO\(_4\) × 7 H\(_2\)O, 0.2; CaCl\(_2\) × 2 H\(_2\)O, 0.02; KH\(_2\)PO\(_4\), 1; K\(_2\)HPO\(_4\), 1; NH\(_4\)NO\(_3\) × 6 H\(_2\)O, 1 and FeCl\(_3\), 0.05 [11]. For emulsification studies, selected strains were cultivated in nutrient broth (NB) medium with the following composition (g l\(^{-1}\)): glucose, 10; yeast extract, 5; proteose–peptone, 5; and NaCl, 5.

**Identification and phylogenetic affiliation of the included strains**

All strains tested in this study were identified by the analysis of the sequence of the gene encoding 16S rRNA (16S rDNA). Primers, fD1 and rD1 [29], were synthesized by Sigma-Genosis (UK) and were used to amplify almost the full length of 16S rRNA gene from each strain. A fresh cultured colony of each strain grown on TSA medium was lysated by the addition of 20 μl of a mixture of NaOH (0.05 M)–SDS (0.25%, w/v) and then boiled for 15 min. The lysates were adjusted to 200 μl with sterile bidistilled water and centrifuged at 13,000 rpm for 5 min in a tabletop centrifuge.

Cleared lysates (4 μl) were used as a template for amplification. PCR reactions were done adding to the lysate 1 × PCR buffer (GeneCraft, Germany), 1.5 mM MgCl\(_2\) (GeneCraft, Germany), 200 μM dNTPs (Roche Molecular Biochemicals, Germany), 20 pmol of each primer and 1 U of Taq polymerase (GeneCraft, Germany). Final volume of the reaction tubes was adjusted to 50 μl. Reactions were run in a Perkin-Elmer GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, USA). The temperature profile was the one previously described by Vinuesa et al. [28]. The amplification products were purified by agarose gel electrophoresis, followed by extraction with the Qiaex II agarose gel extraction kit (Qiagen, Germany). The nucleotide sequence of the purified bands was determined by the dideoxy chain terminator method, using the ABI-PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, USA) and automated sequencer Applied Biosystems ABI 373 (Applied Biosystems, USA). Custom additional primers were synthesized by Sigma-Genosis (UK) when needed to complete the sequence of the whole DNA fragments.

Sequence data were analysed using the GCG Wisconsin Package v. 10.1 programs (Genetics Computing Group, Madison, WI, USA). The BLASTN [2] and FASTA v. 3.3t07 [18] programs were used for preliminary sequence identity analysis. The Clustal X v. 1.81 program [14] was used for sequence alignment, generation of the distance matrix, and construction of

Microbial growth in the presence of PAHs

For construction of growth curves, pure isolates were cultured at 32 °C for 0, 24, 48 and 72 h under aerobic conditions (rotatory shaker, 150 rpm) in liquid BH medium amended with 0% or 0.1% w/v of hydrocarbons. Overnight culture of each bacterial strain grown in NB was harvested by centrifugation (10,000 g, 10 min) and resuspended in sterile phosphate buffer (150 mM, pH 7) to yield an optical density of 0.6 at 660 nm. Aliquots (0.5 ml) of the cell suspensions were transferred to 250 ml Erlenmeyer flasks containing 50 ml of BH medium amended with naphthalene, phenanthrene, fluoroanthene or pyrene. Platable cell counts were estimated by the dilution plate technique (three replicates from each dilution) using TSA (Difco). The inoculated agar plates were incubated at 32 °C for 72 h before colonies were counted.

Biodegradation studies

Naphthalene, phenanthrene, fluoroanthene and pyrene utilization by selected strains in BH medium were assayed by high performance liquid chromatography using a Hewlett-Packard 1050 instrument with an UV Diode Array detector. Samples (50 ml) from inoculated and control (lacking bacteria) were cleared after the addition of 25 ml of methanol, ultrasonic treatment (5 min) and centrifugation at 5000 rpm for 30 min. The determination of naphthalene, phenanthrene, fluoroanthene and pyrene was obtained by the injection of the supernatant in Spherisorb ODSII column (250 x 4.6 mm i.d.), using an isocratic elution with a mixture of 80% acetonitrile and 20% water at a flow rate of 1 ml min⁻¹.

Preliminary characterization and biopolymer production

Production of exopolymers with biosurfactant or bioemulsifier activities by the selected strains was studied according to methods previously reported by Quesada et al. [21] and Scheibenbogen et al. [25]. Erlenmeyer flasks of 500 ml containing 100 ml of NB medium were inoculated with 1 ml of a 24 h culture of microorganisms grown in the same medium. After incubation at 32 °C for 8 days, the cultures were centrifuged at 36,000 g in a Sorval RC-5B refrigerated centrifuge at 4 °C for 60 min. Supernatants obtained were precipitate either with cold ethanol for the isolation of exopolysaccharides [21] or with HCl at pH 2 for the isolation of rhamnolipids [25]. The biopolymers of the precipitated were dissolved in distilled water, dialysed

Fig. 1. Neighbour-joining phylogenetic tree based on the nearly full length of the 16S rDNA gene of the 13 Gram-positive strains described in this paper and sequences from EMBL that gave the highest scores in similarity searches. Nodes highlighted with circles indicate more than 50% of bootstrap value. The accession numbers of the sequences retrieved from EMBL are as follows: Arthrobacter aurescens strain DSM 20116 (X83405), Micrococcus sp. strain LM6-19421 (AJ276811), M. luteus strain HN2-11 (DAF057289), Bacillus pumilus strain KL-052 (AY030327), B. pumilus strain M1-9-1 (AB048252), Bacillus sp. strain 19499 (AJ315067), B. subtilis strain 168 (Z99104), B. subtilis strain DSM-10 (AJ276351), B. licheniformis strain KL-164 (AF391127).
against distilled water during 24 h, lyophilized, and then weighed.

**Emulsification assays and surfactant activity test**

The emulsifying activity of the biopolymers synthesized by the selected strains grown in NB liquid medium was detected by a modified version of the method previously described by Cooper and Goldenberg [10]. Test tubes (105 x 15 mm) were amended with 3.0 ml of exopolymer diluted in distilled water (0.1%, w/v) and 3 ml of a hydrophobic substrate (n-octane, xylene, toluene, mineral oil or crude oil). Then the tubes were shaken vigorously to homogeneity using a vortex, and left to stand for 24 h. Emulsifying activity was expressed as the percentage of the total height occupied by the emulsion. The surface tension of the produced biopolymers was measured with a Krüss K11 digital tensiometer, using a plate method [4].

**Results and discussion**

Bioremediation of areas contaminated with crude, fuel or other hydrocarbon compounds is feasible due to their biodegradability and the diversity of degrading microorganisms present in these sites. Hydrocarbon degrading bacteria are widely spread in polluted soil and water, and research has shown that application of hydrocarbon increases the number of bacteria [4,8,16,32]. Bearing in mind this well-known feature, we have characterized different bacterial strains, which were previously isolated from solid waste oil, with the capacity to grow on culture media supplemented with PAHs. These selected strains included a diversity of Gram-negative and Gram-positive bacteria with the capacity to grow on solid and liquid media amended with naphthalene, phenanthrene, fluoranthene or pyrene as carbon and energy source.

The strategy used to sequence the 16S rDNA gene of each of the strains produced a continuous stretch of >1400 nucleotides, representing >95% of the primary 16S rDNA sequence. Percentage similarity values were obtained after pairwise alignment of the sequences of 16S rDNA of the strains and EMBL database sequences, and the sequences giving the highest scores were retrieved to construct the phylogenetics trees. The strategy used to sequence the 16S rDNA gene of the two Gram-negative strains described in this paper and sequences from EMBL that gave the highest scores in similarity searches. Nodes highlighted with circles indicate more than 50% of bootstrap value. The accession numbers of the sequences retrieved from EMBL are as follows: Alcaligenes defragrans strain 54Pin (AJ005447), A. faecalis strain M3A (AF155147), A. faecalis strain IAM 12369 (D88008), A. faecalis (AJ277669), uncultured soil bacteria clone 816-1 (AF423294), uncultured soil bacteria clone 431-1 (AF423262), uncultured soil bacteria clone cslm2118 (AY133084), uncultured soil bacteria clone 336-1 (AF423250), uncultured soil bacteria clone 1389-1 (AF423223), Enterobacter cloacae strain Nr3 (ECY17665), E. aerogenes strain NCTC10006 T (AJ251468), E. aerogenes (AF395913), Citrobacter freundii strain CDC 621-64 (AF025365), Salmonella enterica serovar typhi strain CT18 (AL627280), S. thyphimurium strain LT2 (AE08706).

**Fig. 2.** Neighbour-joining phylogenetic tree based on the nearly full length of the 16S rDNA gene of the two Gram-negative strains described in this paper and sequences from EMBL that gave the highest scores in similarity searches. Nodes highlighted with circles indicate more than 50% of bootstrap value. The accession numbers of the sequences retrieved from EMBL are as follows: Alcaligenes defragrans strain 54Pin (AJ005447), A. faecalis strain M3A (AF155147), A. faecalis strain IAM 12369 (D88008), A. faecalis (AJ277669), uncultured soil bacteria clone 816-1 (AF423294), uncultured soil bacteria clone 431-1 (AF423262), uncultured soil bacteria clone cslm2118 (AY133084), uncultured soil bacteria clone 336-1 (AF423250), uncultured soil bacteria clone 1389-1 (AF423223), Enterobacter cloacae strain Nr3 (ECY17665), E. aerogenes strain NCTC10006 T (AJ251468), E. aerogenes (AF395913), Citrobacter freundii strain CDC 621-64 (AF025365), Salmonella enterica serovar typhi strain CT18 (AL627280), S. thyphimurium strain LT2 (AE08706).
PAHs are organic compounds that constitute an important fraction of petroleum hydrocarbons, and they are widely distributed in diverse environments. They are exceedingly recalcitrant to degradation due to their inhibitory nature and their very low aqueous solubility [30]. Table 1 summarizes the capacity of selected strains to grow in BH liquid media amended with 0.1% (w/v) of naphthalene, phenanthrene, fluoranthene or pyrene. Positive results have been considered when the viable counts indicated a growth higher than one logarithm. All strains assayed under our experimental conditions grew with naphthalene as carbon source, but none of these strains were able to use the four hydrocarbons tested single-handedly in our study, as a substrate to support their growth. Fluoranthene was used by A. faecalis 212-2. Pyrene was utilized by B. pumilus strains 96-1, 96-6, 96-7, 28-11 and 212-1, and phenanthrene was utilized as sole carbon source by strains of B. pumilus, B. subtilis and M. luteus strains.

The growth curves of the 15 bacterial strains cultured in BH liquid media amended with 0.1% (w/v) of naphthalene, phenanthrene, fluoranthene or pyrene showed that B. pumilus strains grow better on polycyclic aromatic compounds compared with strains of Micrococcus, Alcaligenes and Enterobacter assayed in our experiments. Also, our results showed that phenanthrene was the best substrate to support bacterial growth independently of the strain tested. Fig. 3 shows the growth of B. pumilus strains 96-6, 96-7 and 212-1 in naphthalene, phenanthrene and pyrene, and Fig. 4 shows the growth curves on naphthalene and phenanthrene of B. pumilus 96-4, B. pumilus 28-11 and B. subtilis 28-15.

Table 1. Capacity of isolated strains to grow in BH liquid media with hydrocarbons (naphthalene, phenanthrene, fluoranthene or pyrene) as sole carbon and energy source, and to synthesize extracellular biopolymers in BN liquid medium

<table>
<thead>
<tr>
<th>Strain reference</th>
<th>Growth in hydrocarbon liquid media</th>
<th>Polymer production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Naphthalene</td>
<td>Phenanthrene</td>
</tr>
<tr>
<td>Bacillus pumilus 96-1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus pumilus 96-4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus pumilus 96-6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus pumilus 96-7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus pumilus 96-14</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus pumilus 28-11</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus subtilis 28-15</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Bacillus pumilus 27-1</td>
<td>+</td>
<td>-</td>
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<td>Bacillus subtilis 27-10</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus pumilus 212-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Micrococcus luteus 27-12</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Micrococcus luteus 27-3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Micrococcus luteus 212-4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Alcaligenes faecalis 212-2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter sp. 214-6</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+, Indicates a growth higher than one logarithm determined by enumeration of viable cells.
Naphthalene was utilized for all the selected strains (B. pumilus, B. subtilis, M. luteus, A. faecalis and Enterobacter sp.), whereas fluoranthene was only utilized by one strain affiliated to A. faecalis. Pyrene-removing bacteria were only found in the genus Bacillus, a microbial group that also showed an increased phenanthrene-removal capacity (Table 2). Although no radiolabelled studies with PAH have been performed in our studies in order to demonstrate evolution of $^{14}$CO$_2$ as evidence of mineralization, our results suggest that strains mentioned above posses optimal growth capacities for removing selected hydrocarbons from the

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**Fig. 3.** Growth of *Bacillus pumilus* 96-6 (a), *B. pumilus* 96-7 (b) and *B. pumilus* 212-1(c) in BH liquid medium with naphthalene (■), phenanthrene (▲) and pyrene (×). Growth of bacteria in media without hydrocarbon (-----). Growth of bacteria in media amended with hydrocarbon (—). Values are mean ± standard error of three replicates ($p$ ≤ 0.05).

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**Fig. 4.** Growth of *Bacillus pumilus* 96-4 (a), *B. pumilus* 28-11 (b) and *Bacillus subtilis* 28-15 (c) in BH liquid medium with naphthalene (■) and phenanthrene (▲). Growth of bacteria in media without hydrocarbon (-----). Growth of bacteria in media amended with hydrocarbon (—). Values are mean ± standard error of three replicates ($p$ ≤ 0.05).
growth media under the described experimental conditions. However, PAH utilization seems to be under the control of inducible enzyme systems [19] and obviously a variety of factors can affect the ability of these microorganisms to degraded hydrocarbons in natural environments. In this context, further experiments are needed.

It is well known that microorganisms growing on hydrocarbons frequently produce biopolymers with emulsifying or surfactant activity [11,17,23]. This property is considered as a biological strategy to facilitate the availability of hydrophobic substrates. They can stimulate the growth of hydrocarbon degrading bacteria and improve their ability to utilize these compounds [23]. In this study, assays were carried out to know the capacity of the characterized bacteria to produce extracellular biopolymers with biosurfactant or bioemulsifier activities. Our data show that four strains of the selected microorganisms able to grow on aromatic compounds (PAH) were also able to produce extracellular polymers with high emulsifying activity (Table 1). However, none of the isolated biopolymers showed biosurfactant activity. Some hydrocarbon degrading bacteria produce polymers that primarily act as emulsion stabilizers but usually do not affect the surface tension. Biosurfactants can be of low molecular weight, acting by decreasing the oil–water interfacial tension, or high molecular weight and act as biodispersants by preventing coalescence of oil drops in water. The high molecular weight bioemulsifiers are heteropolysaccharides, and the active components are lipids or proteins. When a preliminary characterization of the exopolymers was performed for the strains B. pumilus 28-15, A. faecalis 212-2, M. luteus 212-4 and Enterobacter sp. 214-6, it was found that this water soluble substances contain high amounts of proteins and carbohydrate (Table 3). However, M. luteus 212-4 produced an extracellular polymer with a high amount of protein and low amount of carbohydrate (56.0% and 3.5% of the total weight of the polymer, respectively). Therefore, our results show that M. luteus 212-4 produce extracellular polymers that can be precipitated by the addition of HCl at pH 2.0, a methodology that has been reported for the isolation of rhamnolipids [25]. In this context, our results suggest that the extracellular polymer produced by M. luteus 212-4 can contain rhamnolipids as a significant component in its chemical composition. However, a final conclusion of this point has not been determined yet.

In conclusion, our study showed that selected strains characterized as B. pumilus, B. subtilis, M. luteus,
A. faecalis and Enterobacter sp. were able to grow and utilize very efficiently PAH such as naphthalene, phenanthrene, fluorene and pyrene. Consequently, our data suggest that these microorganisms could be useful for their application in augmentation and bioremediation technologies.

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