

Isolation and Characterization of a Heterotrophic Bacterium Able to Grow in Different Environmental Stress Conditions, Including Crude Oil and Heavy Metals

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Concentrations of high toxic pollutants are an ever-increasing factor in current soil and aquatic habitats. The isolation of microorganisms having a detoxification capacity is therefore of great interest.

Our research group has isolated a bacterial strain DE2007 from a *Microcoleus* consortium able to degrade crude oil. Cells of strain DE2007 were aerobic, gram negative coccoids, 1µm x 1µm in size, non-motile, encapsulated and non-spore-forming. Colonies grew in LB agar and were cream, smooth, low convex and circular. Strain DE2007 was hetero-organotrophic and quimiolitotrophic and was able to use nitrate as an electron acceptor. Growth occurred in 8°C, 27°C and 43°C (optimum 27°C) and at pH between 3 to 11 (optimum 5-9). The strain could grow in up to 7 % NaCl (w/v) (optimum 1-5 % NaCl). Analysis of the 16SrRNA sequence of strain DE2007 showed it to be a member of the α -3-subclass of the *Proteobacteria*, forming a cluster with the species of the genus *Paracoccus*.

Furthermore, the DE2007 strain is able to grow in the presence of high toxic pollutants such as crude oil (Casablanca and Maya oil) and heavy metals (Cu and Pb).

Keywords: Strain DE2007, crude oil, heavy metals, SEM, TEM.

1. Introduction

Numerous reports of the effects of crude oil and heavy metals (high toxic pollutants) on bacteria from soils, sediments and aquatic habitats have been published [6,10]

Crude oil is a complex mixture of thousand of compounds that can potentially be degraded by a great variety of soil and aquatic microorganisms [17]. The mechanisms by which microorganisms take up hydrocarbons are still far from clear, although it has been established that such compounds enter the cells as intact molecules [16]. Petroleum can sometimes induce accumulation of high electron density (HE) inclusions inside the bacteria; the presence of these inclusions might be considered to indicate ecotoxicity in coastal sediments [9]

On the other hand, the contamination of different habitats by heavy metals is a world-wide problem that still requires an effective technological solution. Hernández E. and Olguín E. J. [13] suggest that biosorption is a promising metal-removal technology. Biosorption is defined as the adsorption capacity of metal compounds from polluted habitats by biological material such as exopolysaccharide (EPS).

Microbial mats are stratified benthonic ecosystems located in coastal sites [11,20] and are sometimes exposed to both types of pollutants. The microorganisms that compose microbial mats are mainly cyanobacteria and have aroused the interest in scientifics since they were developed extensively after oil spills such as those seen in Kuwait following the first Gulf war [1,2]. This microorganism's behavior could indicate a high tolerance of extreme environmental conditions.

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Our research team has studied the diversity and biomass of cyanobacteria in oil-polluted and unpolluted microbial mats around the Europe [7, 19]. We have also isolated a *Microcoleus* consortium, from artificial microbial mats ecosystems (microcosms) [15], able to degrade crude oil. The consortium is formed mainly by a single cyanobacterium, *Microcoleus chthonoplastes*, and by different heterotrophic bacteria [8,18] (Figure 1). In addition, the *Microcoleus* consortium is able to degrade crude oil and has mainly been involved in the degradation of aliphatic heterocyclic organo-sulphur compounds, such as alkylthiolanes and alkylthianes, from Maya oil (sulphur-rich petroleum). Some compounds less harmful from Casablanca oil (high in aliphatic-hydrocarbon content) are also degraded [12]

In this study, we have isolated a heterotrophic bacterium (DE2007) from the above-mentioned consortium. The goals of this work are to characterize this bacterium by microbiological and biochemical techniques and to analyze the effect of petroleum (Casablanca and Maya oils) and heavy metals (Cu and Pb) on bacterial strain DE2007 by means of electron microscopy techniques.

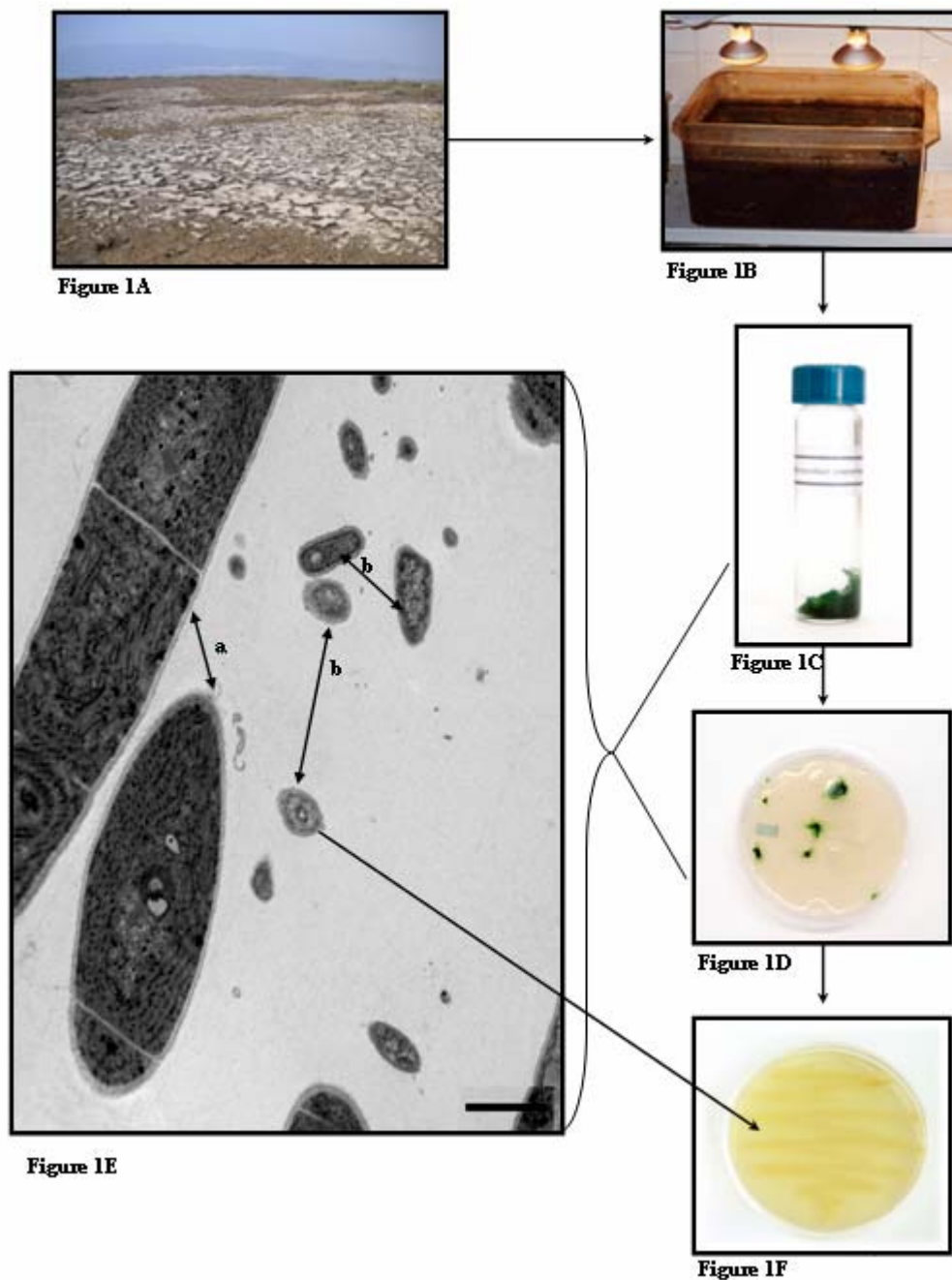


Fig. 1: Microbial mats from the Ebro delta (Tarragona-Spain) (A), Microcosm (artificial ecosystem) (B). *Microcoleus* consortium growing in liquid Mineral Pfenning Medium (C) *Microcoleus* consortium growing in solid Mineral Medium Pfenning (D) Ultrathin section of a *Microcoleus* consortium, showing cyanobacterium filaments (a) and different heterotrophic bacteria (b). Scale bar indicates 1 μ m. (E) Bacterial strain DE2007 growing in LB agar isolated from *Microcoleus* consortium (F).

2. Methods

2.1. Isolation procedure, characterization and identification of strain DE2007

Inoculums from a *Microcoleus* consortium [8] were transferred to Luria-Bertani (LB) medium containing tryptone (10.0 gL^{-1}), yeast extract (5.0 gL^{-1}) and NaCl (10.0 gL^{-1}), pH 7.0. For LB solid medium, 20 g of agar was added. The cultures were incubated in darkness at 27°C . Colonies obtained were streaked on LB agar and isolated in pure culture. The morphological characteristics of strain DE2007 were examined with an Olympus BH2 conventional light microscope. Different stains methods were employed to characterize the DE2007 bacterium (Gram, Negative and Wirtz-Conklin stains). Catalase activity was determined by the presence of bubbles in a 3% H_2O_2 solution [21]. Oxidase activity was analyzed by oxidation of 1% *p*-aminodimethylaniline oxalate. Motility was determined with an optical microscope using the hanging-drop technique. Starch hydrolysis was analyzed as described by Cowan and Steel [3]

Different antibiotics such as ampicilin ($10\mu\text{g}$), tetracycline ($30\mu\text{g}$), streptomycin ($10\mu\text{g}$), kanamycin ($30\mu\text{g}$), erythromycin ($30\mu\text{g}$), chloramphenicol ($30\mu\text{g}$), rifampicin ($30\mu\text{g}$), trimethoprim ($5\mu\text{g}$), amikacin ($30\mu\text{g}$), cefepin ($30\mu\text{g}$), cefuroxin ($30\mu\text{g}$), trobamycin ($10\mu\text{g}$), acid nalidixic ($30\mu\text{g}$), netilmycin ($30\mu\text{g}$) and neomycin ($30\mu\text{g}$) were tested to determine the sensibility of strain DE2007 to distinct antimicrobial agents.

The biochemical assays were made using the API 20 NE strip-identification system (BioMerieux, Marcy l'Étoile, France).

2.2. Conditions of growth

Different environmental stress conditions were tested on bacterial strain DE2007. The pH range for growth was determined by incubating cells in LB medium at 27°C for 4 days at the following pH: 3, 4 (LB broth medium), 5, 6, 7, 8, 9, 10 and 11 (LB agar medium). NaCl tolerance was measured in LB agar medium at concentrations of 0-8 % (w/v). Different temperatures were tested to determine the optimal growth of strain DE2007. All cultures were incubated at 27°C .

To test the ability of strain DE2007 to grow in the presence of petroleum, LB agar plates were covered with 500 μl of Maya or Casablanca oil. To test the ability for growth of strain DE2007 in the presence of heavy metals, different concentrations of Cu and Pb (5mM, 25mM and 50mM) were assayed. All the cultures were incubated at 27°C for 24-48 h.

2.3. Electron microscopy techniques

For Scanning Electron Microscopy (SEM), samples were grown in an LB broth at 27°C , on a rotary shaker (180 r.p.m.), for 48 hours; these were then filtered in nucleopore filters and fixed in 2.5% of glutaraldehyde and washed in buffer phosphate. Subsequently, the samples were dehydrated in increasing concentrations of acetone (30, 50, 70, 90 and 100%) and dried by critical-point drying. Finally, all samples were mounted in metal stubs coated with 96 nm layer of gold. The images were viewed with a Hitachi S-570 scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

For Transmission Electron Microscopy (TEM), samples were fixed with 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde (EM grade, TAAB) in 100 mM phosphate buffer (PB, pH 7.4) for 2 h in an orbital shaker, scrapped, centrifuged at 5000r.p.m. (10 minutes) and rinsed with 100 mM PB. Pellets were then postfixed in 1% (w/v) osmium tetroxide (TAAB) containing 0.8% (w/v) of potassium hexacyanoferrate (III) (Sigma) for 2 h and washed with 100 mM PB. All these steps were carried out at 4°C . Samples were dehydrated through a graded ethanol series, infiltrated in Spurr's resin and polymerized for 48 h at 60°C . Ultrathin sections were mounted in copper grids, contrasted with standard uranyl acetate and Reynolds lead citrate and viewed in a Hitachi H-7000 Transmission Electron Microscopy at 75Kv (Hitachi Ltd. Tokyo, Japan).

3.Results and Discussion

The DE2007 strain was isolated from the *Microcoleus* consortium capable of degrading crude oil. The procedure for the isolation and culture of this strain is shown in Figure 1.

Cells of strain DE2007 were aerobic, Gram-negative coccoids, 1 µm x 1 µm in size, non-motile, encapsulated and non-spore-forming. Colonies grew in LB agar and were cream, low convex, smooth and circular. Growth occurred in 8°, 27° and 43°C (optimum 27°C) and at pH 3 to 11 (optimum 6-8). The optimum NaCl concentration for growth is 1-5 % (w/v). These results are therefore similar to those obtained by Lee et al. [14]. No growth occurs in the presence of more than 8%. The results indicate that DE2007 bacterium is able to grow in different environmental stress conditions.

The biochemical assays demonstrated that strain DE2007 hydrolyzes glucose, arabinose, mannose, maltose, acid adipic, acid malic, tributirine and trisodium citrate, even after 8 days of growth (see table 1). Starch cannot be hydrolyzed. The strain can reduce the nitrates to nitrites, but denitrification is not produced.

Table 1: Features of Strain DE2007.

CHARACTERISTICS	RESULTS
Motility	-
Flagella	-
Capsule	+
Spores	-
Reduction of nitrates to nitrites	+
Reduction of nitrites to nitrogen	-
Indol	-
D-glucose	+
L-arabinose	+
D-mannose	+
D-manitol	-
N-acetyl-glucosamine	-
D-maltosa	+(6 d*)
Potassium gluconate	-
Capric acid	-
Adipic acid	+(8 d*)
Malic acid	+
Trisodium citrate	+(6 d*)
Phenylacetic acid	-
Oxidase	+
Catalase	+
Urea	+
Hydrolysis of esculin	-
Hydrolysis of arginina	-
Hydrolysis of gelatin	-
Hydrolysis of tributirin	+
Hydrolysis of starch	-
H ₂ S production	-
Oxidation/Fermentation	○

* days required for *Paracoccus* sp. growth

In addition, electron microscopy techniques (SEM and TEM) were used to determine morphological parameters. The images obtained by SEM indicate that the bacterium occurs in pairs and did not have flagella (Figure 2A). Ultrathin sections show the characteristic cell wall of Gram negative bacterium (Figure 2B and 2C).

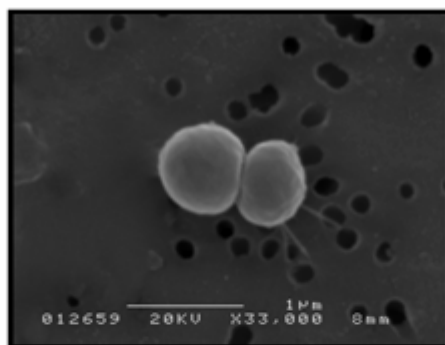


Figure 2A

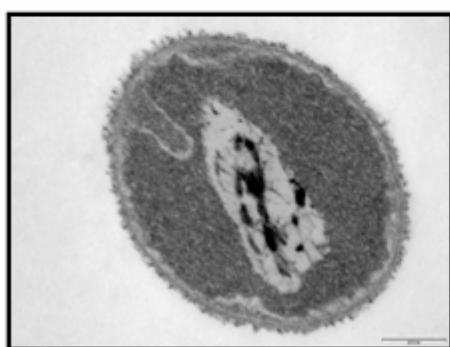


Figure 2B

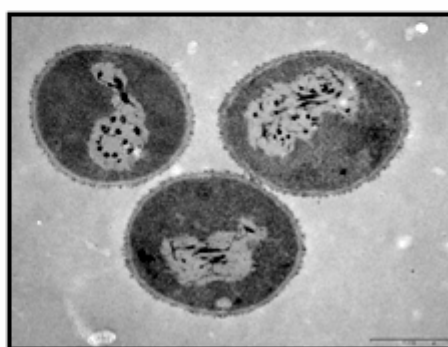


Figure 2C

Fig. 2: Scanning-electron micrograph of control *Paracoccus* sp. (A). Ultrathin sections of strain DE2007 from pristine cultures (B and C). Scale bar indicates 200 and 500 nm, respectively.

Molecular techniques (Polymerase Chain Reaction in Denaturalizing Gradient Gel Electrophoresis) were applied to identifying strain DE2007. Phylogenetic analysis was carried out based on the 16SrRNA gene sequence for strain DE2007; the analysis showed that the bacterium falls into the α -3-subclass of the *Proteobacteria* and forms a cluster with the species of the genus *Paracoccus* (personal communication from María Jesús Pujalte).

Strain DE2007 is susceptible to ampicilin, tetracycline, streptomycin, kanamycin, erythromycin, chloramphenicol and rifampicin, but is resistant to trimethoprim. These results were similar to those obtained for *Paracoccus denitrificans* [22], but strain DE2007 is not a denitrify bacterium. This bacterium is also susceptible to other antibiotics such as amikacin, cefepin, cefuroxin, acid nalidixic, netilmycin, neomycin and trobamycin.

Strain DE2007 grows in the presence of Maya and Casablanca crude oil (Figure 3A and 3B). Ultrathin sections (Figure 3C and 3D) show highly electrondense (HE) inclusions of different sizes distributed throughout the cytoplasm of the bacterium. The number of inclusions was higher in cells growing in Maya oil than in Casablanca; this is probably due to Maya oil being rich in sulphur and aromatic compounds, and in general being more toxic for bacteria. These inclusions have the same features of HE inclusions observed in *Microcoleus chthonoplastes* [9] in similar experiments. The ultrastructure of strain DE2007 shows clear differences between cells growing with and without crude oil. Presence of these inclusions might be considered to indicate ecotoxicity in different oil-polluted habitats.

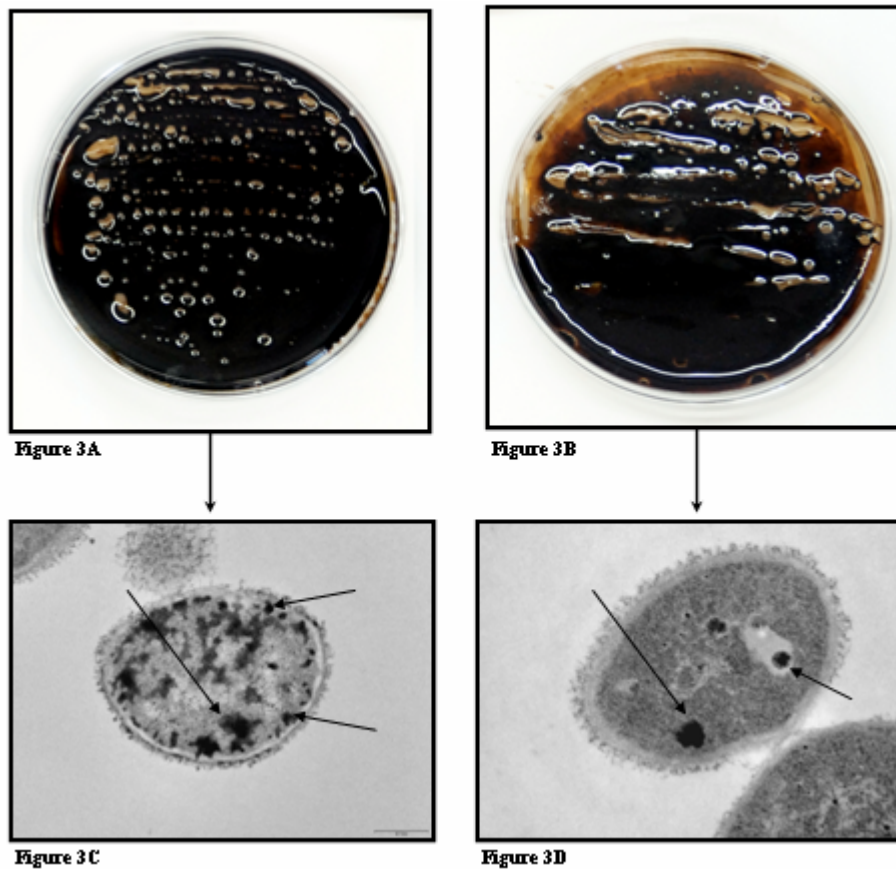


Fig. 3: Bacterial strain DE2007 growing in LB-Maya and Casablanca oil cultures (**A and B**). HE inclusions are indicated by arrows (**C and D**). Scale bar indicates 200 nm.

Strain DE2007 also grows in a heavy-metal polluted LB medium. The ultrathin sections obtained from this bacterium growing in the presence of Pb and Cu is shown in Figure 4. Greater excretion of EPS and vesicles inside the cells can be seen in the above-mentioned conditions. Decho A. W: [4] outlines how EPS can protect cells against potentially toxic compounds by binding these within the exopolysaccharide. Exopolymers could bind transition metals, such as Cu, Cd, Pb, Ag, Fe, Co, Ni, many of which can be toxic to cells. The presence of these metals often triggers excessive exopolymer excretion [5]



Figure 4A



Figure 4B

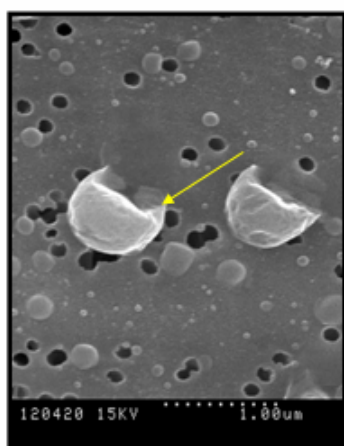


Figure 4C

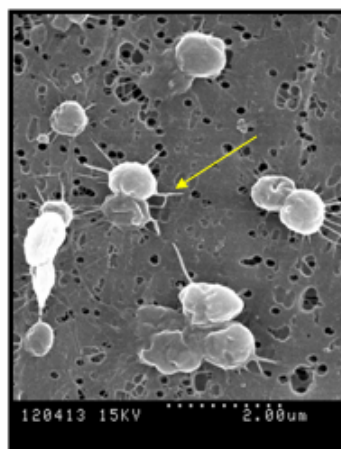


Figure 4D



Figure 4E

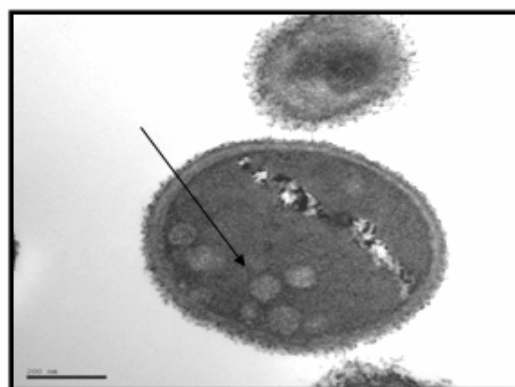


Figure 4F

Fig. 4: Strain DE2007 growing in LB-5mMCuSO₄ (A) and growing in LB-50mMPbSO₄ (B). Scanning-electron micrograph of this bacterium from colonies growing in LB-5mMCuSO₄ (C). Scanning electron micrograph of the strain from colonies growing in LB-50mMPbSO₄ (D). In both images, EPS is indicated by arrows. Scale bar indicates 1 and 2 μm, respectively. Ultrathin section of the heterotrophic bacterium from colonies growing in LB-5mMCuSO₄ (E) and from colonies growing in LB-50mMPbSO₄ (F). Vesicles are indicated by arrows. Scale bar indicates 500 and 200 nm, respectively.

In conclusion, we have isolated a bacterial strain from a consortium of microorganisms able to degrade crude oil.

The DE2007 strain has been characterized and identified as *Paracoccus* sp. in accordance with its morphological, biochemical and molecular characteristics. Furthermore, the strain is able to grow in extreme conditions such as acid and alkaline pH, high salinity, high and low temperature, and in the presence of petroleum and heavy metals (Cu and Pb).

We are currently attempting to determine whether the strain can degrade crude oil, and also to ascertain its capacity for the biosorption of heavy metals.

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