Isolation and characterization of new marine oil-degrading bacteria

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Abstract
The aim of this study was isolation and characterization of new marine oil-degrading bacteria from a seawater sample. A new marine oil-degrading consortium (CtOD) was isolated from Constanta seawater using 5% (v/v) fuel oil, as the sole carbon source. Two bacteria, strains IBB_Ct6 and IBB_Ct7 were isolated from the CtOD consortium. The analysis of the 16S rRNA gene sequences located strains IBB_Ct6 and IBB_Ct7 within genus Pseudomonas, showing 99% similarity to other Pseudomonas strains. The phylogenetic tree was achieved for P. aeruginosa IBB_Ct6 and IBB_Ct7 by performing neighbor-joining analysis of 16S rRNA gene sequences. P. aeruginosa IBB_Ct6 and IBB_Ct7 had distinct metabolic and genomic fingerprinting, compared with CtOD consortium. In the cell-free culture broths of CtOD consortium, P. aeruginosa IBB_Ct6 and IBB_Ct7 the same fractions of biosurfactants were detected in the absence of the hydrocarbons. The biosurfactants produced by these marine bacteria could play an important role in biofilm formation. The CtOD consortium was more resistant to saturated and aromatic hydrocarbons, compared with P. aeruginosa IBB_Ct6 and IBB_Ct7. The high resistance of these marine bacteria against different toxic hydrocarbons could be due to the biosurfactants production and the existence of some catabolic genes (alkB1, alkM1, todCI, todM, xylM, ndoM).

Keywords: oil-degrading consortium, bacteria, biodegradation, hydrocarbons.

1. Introduction
Similar to the microbially mediated breakdown of natural organic matter, biodegradation mediated by indigenous bacterial communities is the ultimate fate of the majority of oil hydrocarbons that enters the marine environment (J.E. KOSTKA & al. [1]). An understanding of the oil impacts on indigenous bacterial communities and identification of the key oil-degrading bacterial groups are prerequisite for directing the management and cleanup of polluted marine environments (J.E. KOSTKA & al. [1]). Oil-degrading bacteria, especially members of the Pseudomonas genus, are ubiquitous in the marine environment, and biodegradation was shown to be successful in naturally remediating oil contamination associated with several spills that impacted different marine environments (J.E. KOSTKA & al. [1]; M. MULET & al. [2]). Some commercial products (e.g., Putidoil) containing different Pseudomonas species are already used for bioaugmentation of large-scale environmental biotechnology (M. MULET & al. [2]).

Although bioremediation field trials were often carried out, there is a paucity of information on the indigenous bacterial communities that catalyze oil degradation under in situ conditions at spill sites. Even less information is available on which members of the bacterial community are active in degrading hydrocarbons, and the impact of various environmental parameters in controlling the activities of indigenous oil-degrading bacteria have not been specifically addressed (J.E. KOSTKA & al. [1]). Recent advances in microbiology make it possible to combine molecular and culture-dependent approaches in
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in order to describe the bacterial communities established in different environments and at different time intervals after the oil spill (J. ALONSO-GUTIÉRREZ & al. [3]). Molecular methods are useful for describing community structure, while bacterial isolation of the key oil-degrading species is needed to characterize the degradation pathways. Consequently, the application of these techniques can improve our understanding of the composition, phylogeny and physiology of metabolically active members of the bacterial community in the marine polluted environment (J. ALONSO-GUTIÉRREZ & al. [3]; M. MULET & al. [2]). Therefore the present study aimed the (1) isolation of new marine oil-degrading bacteria; (2) phenotypic and (3) molecular characterization of marine oil-degrading bacteria; and (4) investigation of the resistance of isolated bacteria to different toxic hydrocarbons.

2. Materials and Methods

Isolation of marine oil-degrading bacteria. Constanta seawater sample (collected during summer) was used to inoculate an enrichment culture in liquid ASW (artificial seawater) medium (A. SEGURA & al. [4]) and fuel oil (5% v/v) as the sole carbon source. Here, as elsewhere in this work each sample was inoculated in duplicate. After 10 days of incubation at 28°C under fully aerobic conditions (rotary shaking, 200 rpm), 10 ml samples from each replicate were used for further isolation and investigation. Moreover, 1 ml samples from each replicate were centrifuged and pellets were stored at -20°C to promote DNA extraction (L.G. WHYTE & al. [5]) and PCR amplification. The most probable number of oil-degrading bacteria (MPN ml⁻¹) in enrichment cultures was determined using the 96-microwell plate method described by M.M. STANCU & M. GRIFOLL [6]. Growth in 96-microwell plate was scored by using a microplate reader (FLUOstar Omega, BMG Labtech), determining the optical density at 660 nm. Growth was also achieved using INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride] method described by M.M. STANCU & M. GRIFOLL [6]. INT-formazan accumulation in the bacterial cells was determined after 24 hours incubation of the microplate at room temperature. Two marine bacteria were isolated after serial dilution of enrichment cultures and plating on LBASW agar (A. SEGURA & al. [4]).

Phenotypic characterization of marine oil-degrading bacteria.

Metabolic fingerprinting. Gram-stain of the marine oil-degrading bacteria was performed using standard Gram microbiology technique. Nutritional features of the marine oil-degrading bacteria were determined using Biolog GN2 microplates. Biolog GN2 microplates were inoculated as recommended by the manufacturer (Biolog, Inc), and incubated for 24 hours at 28°C. Then the formazan accumulation in the bacterial cells was determined.

Biosurfactants production was analyzed by thin layer chromatography (TLC). Biosurfactants were extracted from the cell-free culture broths with chloroform-methanol (2:1 v/v). The samples were spotted with the help of a Linomat 5 sample applicator (CAMAG), on a 10×10 cm precoated silica gel 60 TLC aluminium sheets (Merck). Then the separation was performed using chloroform-methanol-water (65:15:2 v/v/v/v) mixture as mobile phase (P. DAS & al. [7]). After development, a densitometric scan at 254 nm in a TLC Scanner 4 (CAMAG) was performed for detection and quantification of the biosurfactants.

Biofilms formation was studied using scanning electron microscopy (SEM). Culture broths of oil-degrading bacteria were fixed on SEM holder and gold-coated with a JEOL JFC-1300 auto fine coater, in a deep vacuum. The samples were examined with a JEOL JSM-6610LV scanning electron microscope.
Molecular characterization of marine oil-degrading bacteria.

Genomic fingerprinting. For rep-PCR (repetitive sequence-based PCR) amplification, 1 μl of DNA extract was added to a final volume of 25 μl reaction mixture, containing: 5×GqTaq flexi buffer, MgCl2, dNTP mix, specific primers for genomic fingerprinting (REP 1R-Dt and REP 2-Dt, BOXA 1R, (GTG)5, J. VERSALOVIC & al. [8]), and GoTaq DNA polymerase (Promega). PCR was performed with a Mastercycler pro S (Eppendorf). The PCR program consisted in initial denaturation for 6 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 40 to 52°C for 1 min, and extension step at 65°C for 8 min, and a final extension at 65°C for 16 min. After electrophoretic separation on 1.5% (w/v) TBE agarose gel (J. SAMBROOK & al. [9]) and staining with fast blast DNA stain (Bio-Rad) the rep-PCR products were analyzed.

Detection of 16S rRNA genes and catabolic genes. For PCR amplification of these genes, 1 μl of DNA extract was added to a final volume of 25 μl reaction mixture, containing: 10× PCR Rxn buffer, MgCl2, dNTP mix, specific primers for 16S rRNA genes (27f and 1492r for bacteria, J.R. MARCHESI & al. [10]; 20f and 1492r for archaea, V.J. ORPHAN & al. [11]) or primers for catabolic genes (ALK1-f and ALK1-r, ALK2-f and ALK2-r, ALK3-f and ALK3-r, T. KOHNO & al. [12]; alkM-f, alkM-r, F.J. MÁRQUEZ-ROCHA & al. [13]; todC1-f and todC1-r, L.G. WHYTE & al. [5]; todM-f and todM-r, F.J. MÁRQUEZ-ROCHA & al. [13]; TOL-f and TOL-r, B.R. BALDWIN & al. [14]; 23CAT-f and 23CAT-r, M.B. MESARCH & al. [15]; ndoM-f, ndoM-r, F.J. MÁRQUEZ-ROCHA & al. [13]), and Taq DNA polymerase (Invitrogen). PCR was performed with a Mastercycler pro S (Eppendorf). The PCR program consisted in initial denaturation for 10 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 43 to 62°C for 30 sec, and extension step at 72°C for 2 min, and a final extension at 72°C for 10 min. After electrophoretic separation on 1.5% (w/v) TBE agarose gel (J. SAMBROOK & al. [9]) and staining with fast blast DNA stain (Bio-Rad) the PCR products were analyzed.

The taxonomic affiliation of isolated bacteria was determined on the basis of their 16S rRNA sequence. The almost complete 16S rRNA genes of these strains were amplified using the bacterial universal primers (27f and 1492r, J.R. MARCHESI & al. [10]). PCR amplification was performed as described above. The PCR products were purified by using the DNA clean and concentrator-5 kit (Zymo Research), and sequenced with the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems). The reactions were performed using the amplification primers, 27f and 1492r. The products were purified by using the BigDye X Terminator Purification Kit (Applied Biosystems). Sequencing reactions were obtained with an Applied Biosystems 3500/3500xL genetic analyzer at the Institute of Biology Bucharest of Romanian Academy. DNA sequencing runs were assembled using the BioEdit software. The sequences of isolated bacteria were compared to those in public databases using the BLAST search program. The phylogenetic tree was generated by using neighbor-joining method in MEGA5.1 program (K. TAMURA & al. [16]).

Resistance of marine oil-degrading bacteria to toxic hydrocarbons. The saturated hydrocarbons used were: cyclohexane, n-hexane, n-heptane, n-decane, n-pentadecane, and n-hexadecane. The monoaromatic hydrocarbons used were: benzene, toluene, styrene, xylene isomers, and ethylbenzene. The polyaromatic hydrocarbons used were: naphthalene, 2-methynaphthalene, fluorene, anthracene, and phenanthrene. Culture broths of oil-degrading bacteria (10^7 cells ml^{-1}) were spotted on LB_{ASW} agar (control) and on the same medium supplemented with hydrocarbons in vapor phase, except anthracene and phenanthrene which
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have been incorporating into LB$_{\text{ASW}}$ agar. Petri dishes were sealed and the formation of hydrocarbon-resistant bacterial colonies on the agar was determined after 24 hours incubation at 28°C. The growth on LB$_{\text{ASW}}$ agar was estimated by determining the formation of resistant bacterial colonies and the hydrocarbons resistance is represented by the frequency of colony formation, as compared with that observed in the absence of any hydrocarbon taken as 100%.

Reagents used during this study were procured from Merck, Sigma-Aldrich, Promega, Invitrogen, Fermentas, Zymo Research, Applied Biosystems, Biolog or Bio-Rad Laboratories. The PCR primers were purchased from Biosearch Technologies, Integrated DNA Technologies and Invitrogen.

3. Results and Discussions

Isolation of marine oil-degrading bacteria. Seawater sample was collected from Constanta harbor (Romania), which is the largest harbor on the Black Sea and one of the largest ports in Europe. This harbor like others from Europe has been chronically contaminated with petroleum and petroleum products as a result of shipping activities or accidental spillage. A new marine oil-degrading consortium (CtOD) was isolated from Constanta seawater by enrichment cultures method with 5% (v/v) fuel oil as the sole carbon source. The number of total viable oil-degrading bacteria (MPN ml$^{-1}$) in enrichment cultures was between $10^5$ and $10^8$ (data not shown). Two new bacterial strains, IBBCt6 and IBBCt7, were isolated from the CtOD consortium. Knowledge of bacterial community structure and the response of key microbial players in oil-contaminated environments provide a first glance of the metabolic potential and the physiological mechanisms that might drive hydrocarbon degradation (J.E. KOSTKA & al. [1]).

Phenotypic characterization of marine oil-degrading bacteria.

Metabolic fingerprinting. Because both isolated bacterial strains (IBB$_{\text{Ct6}}$, IBB$_{\text{Ct7}}$) were Gram-negative, the metabolic fingerprinting of isolated oil-degrading bacteria was determined by using Biolog GN2 microplates. The acquired results showed distinct Biolog GN2 fingerprinting between CtOD consortium and strains IBB$_{\text{Ct6}}$ and IBB$_{\text{Ct7}}$.

As determined with the Biolog GN2 microplate, the following 93 compounds were utilized by CtOD consortium: dextrin, glycogen, Tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellubiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, $\alpha$-D-glucose, $m$-inositol, $\alpha$-D-lactose, maltose, D-mannitol, D-mannose, D-melibiose, $\beta$-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, $\alpha$-hydroxybutyric acid, $\beta$-hydroxybutyric acid, $\gamma$-hydroxybutyric acid, $\alpha$-ketobutyric acid, $\alpha$-ketoglutaric acid, $\alpha$-ketovaleric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebamic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycy1-L-aspartic acid, glycy1-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, $\gamma$-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethyl-amine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, D,L-$\alpha$-glycerol phosphate, $\alpha$-D-glucose-1-phosphate, and D-glucose-6-phosphate. Only two compounds were not utilized by CtOD consortium: $\alpha$-cyclodextrin and lactulose.

Strain IBB$_{\text{Ct6}}$ utilized the following 43 compounds: Tween 40, Tween 80, L-arabinose, D-fructose, $\alpha$-D-glucose, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, $\alpha$-hydroxybutyric acid, $\beta$-hydroxybutyric acid, $\gamma$-hydroxybutyric acid, $\alpha$-ketobutyric acid, $\alpha$-ketoglutaric acid, $\alpha$-ketovaleric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebamic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycy1-L-aspartic acid, glycy1-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, $\gamma$-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethyl-amine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, D,L-$\alpha$-glycerol phosphate, $\alpha$-D-glucose-1-phosphate, and D-glucose-6-phosphate. Only two compounds were not utilized by CtOD consortium: $\alpha$-cyclodextrin and lactulose.
acid, cis-aconitic acid, citric acid, formic acid, D-gluconic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, p-hydroxy-phenylacetic acid, itaconic acid, α-ketoglutaric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, succinic acid, bromosuccinic acid, succinamic acid, D-alanine, L-alanine, L-asparagus, L-aspartic acid, L-glutamic acid, L-histidine, hydroxy-L-proline, L-ornithine, L-proline, L-pyrogalacturonic acid, L-serine, D,L-carnitine, γ-aminobutyric acid, urocanic acid, inosine, putrescine, 2-aminoethanol, 2,3-butanediol, and glycerol. The others fifty-two compounds of the Biolog GN2 microplate were not utilized by strain IBBCt6. Based on these metabolic fingerprinting, the strain IBBCt6 shows 100% similarity to other *P. aeruginosa* strains from the Biolog database.

The following 45 compounds were utilized by IBBCt7 strain: Tween 40, Tween 80, L-arabinose, D-fructose, α-D-glucose, D-mannit, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, cis-aconitic acid, citric acid, formic acid, D-gluconic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, p-hydroxy-phenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-pyrogalacturonic acid, L-serine, L-threonine, γ-aminobutyric acid, urocanic acid, putrescine, 2-aminoethanol, 2,3-butanediol, and glycerol. Fifty compounds of the Biolog GN2 microplate were not utilized by strain IBBCt7. Based on their metabolic fingerprinting, the strain IBBCt7 shows 100% similarity to other *P. aeruginosa* strains from the Biolog database.

As observed, *P. aeruginosa* IBBCt6 and *P. aeruginosa* IBBCt7 used a smaller number of Biolog GN2 microplates compounds (i.e., 43 and 45), as compared with CtOD consortium (i.e., 93 compounds). As expected, oil-degrading consortium possesses broad enzymatic equipment, compared with individual bacterial strains.

Biosurfactants production. Thin layer chromatography (TLC) was performed to investigate biosurfactants (surface-active agents) production in marine oil-degrading bacteria (Fig. 1a, b). As evident from TLC analysis, the different biosurfactants fractions (Fig. 1a) were produced by CtOD consortium, *P. aeruginosa* IBBCt6 and IBBCt7, in liquid LBASW medium after 24 hours incubation at 28°C. The same fractions of biosurfactants were detected in enrichment cultures liquid of CtOD consortium grown on ASW medium in the presence of 5% (v/v) fuel oil, as the sole carbon source (data not shown).

**Figure 1:** Biosurfactants production by marine oil-degrading bacteria

The TLC plate was scanned (panel a) and visualized (panel b) under a 254 nm ultraviolet light; CtOD consortium (lane 1), *P. aeruginosa* IBBCt6 (lane 2), *P. aeruginosa* IBBCt7 (lane 3), L-rhamnose (lane 4); the biosurfactants fractions with Rf between 0.48 and 1.06 have been marked by bracket, the biosurfactants spots (panel b) and their corresponding peak (panel a) have been marked by arrows.
Predominantly, biosurfactants are synthesized by a variety of bacteria during growth on water-immiscible substrates, such as hydrocarbons. However, the bacteria isolated in this study were able to produce biosurfactants in the liquid LBASW medium in the absence of the hydrocarbons. These results indicate that hydrocarbons are not essential to these bacteria for biosurfactants production. Biosurfactants fractions produced by CtOD consortium separated on TLC plate at $R_f$ (retardation factor) between 0.48 and 1.05. For $P.\ aeruginosa$ IBB$_{C6}$ and IBB$_{C7}$ the biosurfactants separated at $R_f$ between 0.49 and 1.06. Two distinct biosurfactants spots (Fig. 1b) were observed on the TLC plate visualized under ultraviolet light (254 nm). The $R_f$ values were 0.60-0.61 for the first spot, and 0.71-0.74 for the second spot. Their $R_f$ values were similar to data given in the literature (A.M. ABDEL-MAWGOUD & al. [17]), and these value correspond to rhamnolipids. Most of the biosurfactants produced by different $P.\ aeruginosa$ strains are rhamnolipids (lipids containing rhamnose). These type of biosurfactants are a unique class of amphiphilic compounds that have been shown to have a variety of potential applications in the remediation of hydrocarbon polluted sites, in the enhanced transport of bacteria, in enhanced oil recovery, as cosmetic additives, in biological control, and in medicine for their antimicrobial, antitumoral, antiviral, and anti-inflammatory activities (P. DAS & al. [6]; Soberón-Chávez & al. [18]).

**Biofilms formation.** Biosurfactants produced by $P.\ aeruginosa$ play an important role in structural biofilm development (S.J. PAMP & T. TOLKER-NIELSEN [19]). It has been shown that low concentrations of rhamnolipid enhance the cell surface hydrophobicity of $P.\ aeruginosa$ by causing a release of lipopolysaccharides (LPS) from the cell surface. An increase in cell surface hydrophobicity could increase the adhesiveness of the bacteria to a level which is critical for initial microcolony formation in biofilms (S.J. PAMP & T. TOLKER-NIELSEN [19]). Scanning electron microscopy (SEM) was performed to study biofilms formation by marine oil-degrading bacteria (Fig. 2a-d). SEM studies on CtOD consortium grown on ASW medium in the presence of 5% (v/v) fuel oil revealed biofilms formation. The biofilms formation was also observed when CtOD consortium, $P.\ aeruginosa$ IBB$_{C6}$ and IBB$_{C7}$ were grown on LBASW medium without hydrocarbons. Furthermore, cells grown on LBASW medium were found to be connected to each other maybe due to extracellular material secreted by these bacteria. The formation of the biofilms when these bacteria were grown on LBASW medium (without hydrocarbons) could be due to the production of biosurfactants which enhance the cell surface hydrophobicity. As mentioned above, an increase in the cell surface hydrophobicity promote microcolony formation in the initial phase of biofilm formation.

**Figure 2:** Scanning electron microscopy of marine oil-degrading bacteria

CtOD consortium grown on ASW medium in the presence of 5% (v/v) fuel oil (panel a); CtOD consortium (panel b), $P.\ aeruginosa$ IBB$_{C6}$ (panel c), and $P.\ aeruginosa$ IBB$_{C7}$ (panel d) grown on LBASW medium
Molecular characterization of marine oil-degrading bacteria.

Genomic fingerprinting. Repetitive sequence-based PCR (rep-PCR) has been used previously to generate DNA fingerprints to distinguish between genetically unrelated isolates and closely related bacterial strains (D. BHATTACHARYA & al. [20]). This technique involves the use of specific primers based on the short repetitive elements derived from highly conserved palindromic inverted repeat regions dispersed throughout the prokaryotic kingdom. Amplification of the regions between adjacent repetitive extragenic elements gives strain-specific DNA fingerprints (J. VERSALOVIC & al. [8]; D. BHATTACHARYA et al. [20]). Genotypic relationships among CtOD consortium, *P. aeruginosa* IBB_C6 and IBB_C7 were determined by rep-PCR, using REP, BOXA and (GTG)_5 primers (Fig. 3).

![Genomic fingerprinting of marine oil-degrading bacteria](image)

Amplification using a gradient of annealing temperatures indicated optimal annealing temperatures of 40°C for REP primers, 50°C for BOXA primers, and 52°C for (GTG)_5 primers (data not shown). (GTG)_5 primers produced the most complex amplified banding patterns for marine oil-degrading bacteria, with sizes ranging from 100 bp to 3,000 bp. The amplified banding patterns were 100-2,500 bp and 500-2,000 bp when the primer sets REP and BOXA were used. Based on their genomic fingerprinting, *P. aeruginosa* IBB_C6 and *P. aeruginosa* IBB_C7 were separated into two distinguishable genotypic groups.

Detection of 16S rRNA genes and catabolic genes. Genomic DNA extracted from marine oil-degrading consortium and individual bacterial strains were used as template for PCR amplification of 16S rRNA genes and some catabolic genes.

Analysis of 16S rRNA genes represents a powerful tool to study bacterial community structure in enrichment cultures. Amplification of the expected fragment was observed only for bacterial 16S rRNA genes (1465 bp), whereas the amplification of the expected size fragment for archaeal 16S rRNA genes (1472 bp) was not observed in DNA extracted from CtOD consortium.

The catabolic genes encoding alkane hydroxylase (*alkB, alkM, alkB1, alkM1*), toluene dioxygenase (*todC1, todM*), toluene/xylene monooxygenase (*xylM*), catechol 2,3-dioxygenase (*C23DO*) and naphthalene dioxygenase (*ndoM*) were used to assess the capacity of these bacteria to degrade saturated or aromatic hydrocarbons. The expected PCR products sizes were 185 bp, 271 bp, 330 bp, and 870 bp for alkane hydroxylase genes, 560 bp for toluene dioxygenase, 475 bp for toluene/xylene monooxygenase, 238 bp for catechol 2,3-dioxygenase, and 642 bp for naphthalene dioxygenase genes. Amplification using a gradient of annealing temperatures indicated optimal annealing temperatures of 43°C for ALK1-3 primers, 62°C for alkM, todM, and ndoM primers, 53°C for todC1 and TOL primers, and 56°C for 23CAT primers. In DNA extracted from CtOD consortium were detected four
catabolic genes (i.e., \textit{alkB1}, \textit{todC1}, \textit{xylM}, \textit{ndoM}). Only three catabolic genes were detected in DNA extracted from \textit{P. aeruginosa IBB}_{C6} (i.e., \textit{alkB1}, \textit{alkM1}, \textit{todC1}) and \textit{P. aeruginosa IBB}_{C7} (i.e., \textit{alkB1}, \textit{todC1}, \textit{todM}). Individual bacterial cultures (which metabolize only a limited range of hydrocarbons) showed less growth and degradation of the crude oil, as compared with the mixed bacterial consortium which degraded a wider range of hydrocarbons (R.S. AL-WASIFY & S.R. HAMED [21]). As observed in Table 1 the genes \textit{alkM1} and \textit{todM} were not detected in DNA extracted from CtOD consortium. Though, \textit{alkM1} and \textit{todM} genes were detected in DNA extracted from \textit{P. aeruginosa IBB}_{C6} and \textit{P. aeruginosa IBB}_{C7}, respectively; unspecific amplification of other fragments were observed when the primer sets \textit{alkM} and \textit{todM} were used. Although, the used primers are specific for some catabolic genes previously described (F.J. MÁRQUEZ-ROCHA & al. [13]), they were not specially designed for the bacteria isolated in this study causing unspecific amplification of other fragments.

Table 1: Detection of 16S rRNA and catabolic genes in marine oil-degrading bacteria

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<th>Bacteria</th>
<th>16S rRNA</th>
<th>Catabolic genes</th>
<th>Alkane hydroxylase</th>
<th>Toluene dioxygenase</th>
<th>Toluene/xylene monooxygenase</th>
<th>Catechol 2,3-dioxygenase</th>
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Legend: \textit{f} = forward PCR primer; \textit{r} = reverse PCR primer; \textit{Ta} = annealing temperature (°C); \textit{Fs} = PCR fragment size (bp)

The DNA extracted from CtOD consortium, \textit{P. aeruginosa IBB}_{C6} and \textit{P. aeruginosa IBB}_{C7} were also screened by PCR for the presence of \textit{otsA1} gene using \textit{otsA-f} and \textit{otsA-r} degenerate primers (D. TISCHLER & al. [22]). PCR amplification was performed as described in materials and methods for catabolic genes, except annealing temperature which was 50°C in the case of these primers. \textit{P. aeruginosa IBB}_{C6} and \textit{P. aeruginosa IBB}_{C7} possesses a homologues of \textit{otsA1} gene (760 bp), while in DNA extracted from CtOD consortium this gene was not detected. Furthermore, unspecific amplification of other fragment (560 bp) was observed in CtOD consortium, \textit{P. aeruginosa IBB}_{C6} and \textit{P. aeruginosa IBB}_{C7}. To our knowledge, this is the first observation of \textit{otsA1} gene in \textit{Pseudomonas aeruginosa}. As shown previously, \textit{otsA1} gene seems to be involved in the overproduction of trehalose lipids by \textit{Rhodococcus opacus} 1CP during growth on \textit{n}-alkanes (D. TISCHLER & al. [22]).

\textbf{The taxonomic affiliation.} Detailed genetic analysis at the species level gives insight into the variability within a bacterial population and helps to generate evidence of genome plasticity and evolution, which enable bacterial adaptation to various environmental conditions. The nucleotide base sequence of the gene coding for 16S rRNA is considered an important standard for bacterial identification and for deriving phyllogenetic relationships among different organisms. Bacteria can be identified down to the genus and species level by amplifying and sequencing the 16S rRNA genes and comparing them to the database of known 16S rRNA sequences (D. BHATTACHARYYA & al. [20]). The 16S rRNA genes of strains IBBC_{6} and IBBC_{7} were amplified by a PCR using two bacterial universal primers, and almost-complete nucleotide sequences (1370 bp, 1378 bp) were determined. The analysis of the 16S rRNA gene sequences located strains IBBC_{6} (GenBank JN937659) and IBBC_{7} (GenBank JN937660) within family \textit{Pseudomonadaceae}, genus \textit{Pseudomonas}, showing 99% similarity to other \textit{Pseudomonas} strains from the public databases (GenBank/DDBJ/EMBL) (Fig. 4).
Phylogenetic tree based on 16S rRNA gene sequences, showing the position of *P. aeruginosa* IBBC\textsuperscript{c6} and *P. aeruginosa* IBBC\textsuperscript{c7} with respect to other *Pseudomonas* strains from the public databases.

The scale bar indicates substitutions per nucleotide position.

Phylogenetic tree was inferred by performing neighbor-joining analysis in MEGA5.1 program. In the 16S rRNA gene phylogenetic tree, *P. aeruginosa* IBBC\textsuperscript{c6} formed a cluster with *P. sp. a-1-10*, *P. aeruginosa* ALK\textsuperscript{320}, *P. aeruginosa* ALK\textsuperscript{318}, *P. aeruginosa* ALK\textsuperscript{317}, and *P. aeruginosa* ALK\textsuperscript{316}, whereas *P. aeruginosa* IBBC\textsuperscript{c7} formed a tight cluster with *P. aeruginosa* R1-135 and *P. aeruginosa* LHZ\textsuperscript{008}.

Resistance of marine oil-degrading bacteria to toxic hydrocarbons. The tolerance of bacteria to hydrocarbons is the key factor for hydrocarbons degradation. Therefore, for further characterization of marine oil-degrading bacteria, the tolerance to different toxic saturated hydrocarbons and aromatic hydrocarbons was investigated (Table 2). The CtOD consortium was more resistant (75-100%) to toxic hydrocarbons, as compared with *P. aeruginosa* IBBC\textsuperscript{c6} and IBBC\textsuperscript{c7} (10-100%). Saturated hydrocarbons with higher log *P_{OW}* values (between 3.35 and 9.15) were less toxic for the oil-degrading consortium and individual bacterial strains, though the aromatic hydrocarbons with lower log *P_{OW}* values (between 2.14 and 4.49) were more toxic for these bacteria. Some bacterial strains are able to thrive in the presence of high concentrations of toxic hydrocarbons. According to literature (A. SEGURA & al. [23]), the extrusion of these toxic compounds from the cell to the external medium represents the most relevant aspect in the hydrocarbon tolerance of bacteria. However, hydrocarbon tolerance is a multifactorial process that involves a wide range of genetic and physiological changes to overcome toxic hydrocarbons damage (A. SEGURA & al. [23]).
Isolation and characterization of new marine oil-degrading bacteria

<table>
<thead>
<tr>
<th>Table 2: Resistance of marine oil-degrading bacteria to hydrocarbons</th>
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Legend: \( P_{OW} \) = logarithm of the partition coefficient of the hydrocarbons in octanol-water mixture.

4. Conclusions

A new marine oil-degrading consortium (CtOD) was isolated from Constanta seawater by enrichment cultures method using 5% (v/v) fuel oil as the sole carbon source. Two new bacteria, strains IBB_{Ct6} and IBB_{Ct7} were isolated from the CtOD consortium. The analysis of the 16S rRNA gene sequences located strains IBB_{Ct6} and IBB_{Ct7} within genus *Pseudomonas*, showing 99% similarity to other *Pseudomonas* strains. *P. aeruginosa* IBB_{Ct6} and *P. aeruginosa* IBB_{Ct7} had distinct metabolic and genomic fingerprinting, as compared with CtOD consortium. The same fractions of biosurfactants were detected in the cell-free culture broths of CtOD consortium, *P. aeruginosa* IBB_{Ct6} and *P. aeruginosa* IBB_{Ct7}, in the absence of the hydrocarbons. The production of biosurfactants in the absence of the hydrocarbons is very important for potential applications in the remediation of hydrocarbon polluted sites and also in oil recovery. It is well known that *P. aeruginosa* is a bacterium with remarkable metabolic diversity and this bacterium is able to produce biosurfactants which can enhance not only the bioavailability of fuel components but also the growth of other degrading bacteria. The CtOD consortium was more resistant to saturated and aromatic hydrocarbons, compared with *P. aeruginosa* IBB_{Ct6} and *P. aeruginosa* IBB_{Ct7}, in the absence of the hydrocarbons. The resistance of the CtOD consortium, *P. aeruginosa* IBB_{Ct6} and IBB_{Ct7} against different toxic hydrocarbons could be due to the biosurfactants production and the existence of some catabolic genes. The acquired results suggested that isolated oil-degrading bacteria could play a key role in the *in situ* degradation of the petroleum and petroleum products in the marine environments.

5. Acknowledgements

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