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Biodegradation potentiality of psychrophilic bacterial strain *Oleispira antarctica* RB-8^T

G. Gentile [a,*], M. Bonsignore [a], S. Santisi [a,b], M. Catalfamo [a], L. Giuliano [c], L. Genovese [a], M.M. Yakimov [a], R. Denaro [a], M. Genovese [a], S. Cappello [a]

[a] Institute for Coastal Marine Environment (IAMC)-CNR of Messina, Messina, Italy
[b] Ph.D School in "Biology and Cellular Biotechnology" of University of Messina, Messina, Italy
[c] Institute for Coastal Marine Environment (IAMC)-CNR of Napoli, Naples, Italy

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ABSTRACT

The present study is focused on assessing the growth and hydrocarbon-degrading capability of the psychrophilic strain *Oleispira antarctica* RB-8^T. This study considered six hydrocarbon mixtures that were tested for 22 days at two different cultivation temperatures (4 and 15 °C). During the incubation period, six sub-aliquots of each culture at different times were processed for total bacterial abundance and GC–FID (gas chromatography–flame ionization detection) hydrocarbon analysis.
Results from DNA extraction and DAPI (4′,6-diamidino-2-phenylindole) staining showed a linear increase during the first 18 days of the experiment in almost all the substrates used; both techniques showed a good match, but the difference in values obtained was approximately one order of magnitude. GC–FID results revealed a substantial hydrocarbon degradation rate in almost all hydrocarbon sources and in particular at 15 °C rather than 4 °C (for commercial oil engine, oily waste, fuel jet, and crude oil). A more efficient degradation was observed in cultures grown with diesel and bilge water at 4 °C.

1. Introduction

The relatively recent discovery of a new group of marine hydrocarbon-degrading bacteria (Dyksterhouse et al., 1995; Liu and Shao, 2005; Yakimov et al., 1998, 2003, 2004; Golyshin et al., 2002) revealed the significant role of these microorganisms in solving bioremediation problems in marine environments. Several studies have focused on microbial hydrocarbon biodegradation in cold marine environments (Brakstad and Bonaunet, 2006; Guibert et al., 2012; Rodríguez-Blanco et al., 2013; Muangchinda et al., 2015). In particular, fishing and supply vessels and even cruise ships regularly cross the Antarctic sea, releasing continuously significant amounts of diesel fuel and lubricating oil in the marine environment (Michaud et al., 2004). At low temperatures, biodegradation processes are limited by several factors; for example, an increase in hydrocarbon mixture viscosity reduces the degree of its spreading in water systems. Conversely, short-chain alkanes (<C10), whose volatilization is retarded, become more soluble in the aqueous phase and their toxicity toward microorganisms increases, thereby delaying biodegradation efficiency (Michaud et al., 2004). Marine ship-source oil spills in the Antarctic region can have significant impacts on the marine environment (Cappello et al., 2014), which can severely damage the surrounding ecosystems (Head et al., 2006).

Biodegradation by natural populations of microorganisms is the most reliable mechanism by which thousands of xenobiotic pollutants, including crude oil, are eliminated from the environment (Cappello et al., 2007). The high metabolic potential of microorganisms is currently of great interest in the field of marine biotechnology. According to Yakimov et al. (2007), marine hydrocarbon-degrading bacteria are present at low or undetectable levels before a pollution event; the introduction of oil constituents into seawater leads to successive blooms of a relatively limited number of indigenous marine bacterial genera, the so-called OHCB (obligate hydrocarbonoclastic bacteria). Thus, the utilization of indigenous marine microorganisms in the bioremediation process, which have the ability to survive and continue their activity under in situ conditions, appears fundamental for the removal of hydrocarbons from the environment.

Oleispira antarctica (Yakimov et al., 2003) represents a new genus of hydrocarbon-degrading bacterium isolated from the Antarctic environment despite being found in other cold areas at high latitudes as well (Gerdes et al., 2005; Bissett et al., 2006; Prabagaran et al., 2007). *O. antarctica* RB 8^T strain was first isolated from crude-oil enrichments of Antarctic seawater (Yakimov et al., 2003). Several microcosm studies performed at low temperatures showed the presence of organisms related to *Oleispira* sp., blooming in oil-degrading microbial communities (Coulon et al., 2007). 16S rRNA clones affiliated to the genus *Oleispira* were also found to be predominant in deep-water samples

* Corresponding author at: Institute for Coastal Marine Environment (IAMC)-CNR Spianata S. Raineri, 86-98122 Messina, Italy.
E-mail address: gabriella.gentile@iamc.cnr.it (G. Gentile).

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after blowout of the Deepwater Horizon platform in the Gulf of Mexico (Hazen et al., 2010; Mason et al., 2012).

Oleispira spp. as well as other representatives of the order Oceanospirillales, including the genera *Alcanivorax*, *Thalassolituus*, and *Oleiphilus*, belong to a specific group of the so-called OHCB (Yakimov et al., 2007); their metabolic functions are restricted to the linear and branched aliphatic, saturated and unsaturated hydrocarbons and their derivatives such as fatty acids or alcohols (Yakimov et al., 2007).

Genome sequencing of *O. antarctica* RB8^T revealed three genes for alkane monooxygenases/fatty acid desaturases, which are, on par with *Marinobacter aquaeolei* VT8, most frequently found in marine Gammaproteobacteria, and one P450 cytochrome that is presumably involved in terminal hydroxylation of hydrocarbons (Kube et al., 2013). Genome analysis and genome-based functional studies revealed unique insights into its alkane-degrading ability, thus emphasizing the prominent role of this psychrophilic microorganism in cold and deep marine environments, especially in oil spill events.

On the basis of the literature, this study aims to analyze the ability of *O. antarctica* RB8^T strain to grow and degrade different hydrocarbon mixtures. In particular, bacterial abundance (rate of total DNA amount and DAPI (4′,6-diamidino-2-phenylindole) count) was estimated and analyzed at six successive sampling times and compared with the degradation rate (gas chromatography–flame ionization detection; GC–FID analysis) of different hydrocarbons used in cultures in this study.

2. Material and methods

2.1. Experimental setup and growth conditions

In this study, *O. antarctica* RB8^T strain (AJ426420, DSM N° 14852, Yakimov et al., 2003) was used in all the experiments. The strain used in this study belongs to a collection of hydrocarbon-degrading bacteria held at the Institute for Coastal Marine Environment (IAMC)-CNR of Messina and was isolated originally from hydrocarbon-degrading enrichment cultures obtained from Rod Bay (Ross Sea) in an Antarctic coastal marine environment (Yakimov et al., 2003).

A starter culture was prepared by inoculating a loop of microbial cells into 25 ml of ONR7, a mineral medium (Dyksterhouse et al., 1995) supplemented with 0.5% (*w*/*v*) of sterile tetradecane ($C_{14}H_{30}$, Sigma-Aldrich, Milan, Italy) as a single carbon source. The culture was allowed to grow in a rotary shaker (New Brunswick C24KC, Edison NJ, USA; 200 × *g*) at 4 ± 1 °C for 7 days. After 1 week, 200 μl of the cells in mid-exponential phase were harvested by centrifugation at 9000 × *g* for 10 min, washed twice with sterile medium, and inoculated into 250-ml sterile Erlenmeyer flasks (Cefalì et al., 2002), with each containing 100 ml of ONR7, a medium supplemented with 1% (*w*/*v*) of hydrocarbon source. Different enrichment cultures were carried out using the following: *i*) car commercial diesel (AGIP S.p.A., indicated as DIESEL); *ii*) commercial crude oil engine (indicated as ENG); *iii*, *iv*) saline oily wastes originated from marine transportation (bilge water) from two different ships (named WASTE and BW, respectively); *v*) military jet fuel (Jet Propellant, JP-5; named JP5); and *vi*) crude oil (PierE1, Dansk Crude Oil; indicated as OIL).

Cultures were incubated for 22 days in a rotary shaker (New Brunswick C24KC, Edison, NJ, USA; 200 × *g*) at two different experimental temperatures, 4 ± 1 and 15 ± 1 °C (Yakimov et al., 2003). Abiotic controls (enrichments in sterile conditions without bacterial inoculation) were prepared in parallel in order to evaluate the degradation of each hydrocarbon due to purely physicochemical processes. All experiments were carried out in triplicate for every condition (Yakimov et al., 2003).

2.2. Sampling strategy and parameter assays

During the experimental period (22 days), sub-aliquots of each bacterial culture, at different temperatures, were collected aseptically six times (T_7, T_9, T_{11}, T_{15}, T_{18}, and T_{22}), and totally twelve templates (six at 4 °C and six at 15 °C) were processed for each sampling time.

2.2.1. Total DNA amount

The total amount of DNA was used as an indirect index of bacterial abundance (Genovese et al., 2008). DNA was extracted from 2 ml of each culture using MasterPure™ Complete DNA and RNA Purification Kit (Epicenter). After elution with 35 μl of 1 × TE (Tris and ethylenediaminetetraacetic acid) buffer, nucleic acids were stored at −20 °C. DNA was quantified using a Spectrophotomer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

The relative abundance of bacteria (BA, cell ml^{-1}) was calculated using the following formula:

$$BA = \left(gr_{DNA} \times 6.022 \times 10^{23}\right) / \left(G \times 1x10^{9} \times 660\right)$$

where gr_{DNA} is the DNA amount expressed in grams, 6.022×10^{23} is Avogadro's number (6.022×10^{23} mol^{-1}), 660 is the weight of one mole of a double stranded of a base pair, and G is genome size in base pairs. The genome size of 4,406,383 bp of *O. antarctica* RB-8^T (Kube et al., 2013) was used for calculation.

2.2.2. DAPI staining

In order to evaluate a possible growth correspondence in both analysis techniques, bacterial abundance was evaluated indirectly by analyzing total DNA amount and also by direct counting via DAPI staining. In particular, for DAPI staining, 1 ml of each sample was fixed in formaldehyde (2% final concentration) and stored at 4 °C until analysis. After a short period (30 s) of ultrasonic treatment (Ultrasonic Bath Branson 1200, Branson, USA), the samples were filtered using Nuclepore black polycarbonate filters (pore size: 0.2 μm) and the filters stained with 4, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich S.R.L., Milan, Italy; 5 mg l^{-1} final concentration), according to standard analytical protocols (Porter and Feig, 1980; Cappello et al., 2012). Slides were examined with a Zeiss AXIOPLAN 2 Imaging epifluorescence microscope (Zeiss; Carl Zeiss Inc., Thornwood, NY, USA) and the labeled cells present in at least 30 microscopic fields were counted. Results were expressed as number of cells per milliliter.

2.2.3. Hydrocarbon analysis

The composition of total extracted and resolved hydrocarbons and their derivates (TERHCs) were analyzed by high-resolution GC–FID (DANI Master GC Fast Gas Chromatograph System, DANI Instruments S.p.A., Milan, Italy) following the 3510 EPA (Environmental Protection Agency). After acidification, TERHCs from samples were extracted at room temperature on a shaking table using dichloromethane (CH_2Cl_2, Sigma-Aldrich, Milan, Italy; 10% *v*/v). This procedure was repeated thrice, and the CH_2Cl_2 phase was combined and treated with sodium sulfate anhydrous (Na_2SO_4, Sigma-Aldrich, Milan, Italy) in order to remove any residual water (Ehrhardt et al., 1991; Wang et al., 1998; Dutta and Harayama, 2001; Denaro et al., 2005). The extracts were concentrated by rotary evaporation (Rotavapor model R110; Büchi Labortechnik AG, Switzerland) at room temperature (<30 °C), followed by evaporation under a stream of nitrogen and taken up into a solution containing heptamethyl–nonane as an internal standard (79 μg mL^{-1}). As indicated, all measurements were performed using a Master GC DANI Instruments equipped with a split/splitless SSL injector and FID detector. Samples (1 μl) were injected in the splitless mode at 330 °C. The analytical column was a Restek Rxi-5 Sil MS with Integra-Guard, 30 m × 0.25 mm (ID × 0.25-μm film thickness). Helium carrier gas was maintained at a constant flow of 1.5 ml min^{-1}. TERCHs were calculated with the mean response factors of *n*-alkanes; that is, the individual *n*-alkane concentrations, pristane and phytane, were calculated.