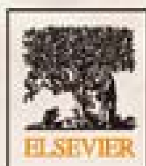
JES

JOURNAL OF ENVIRONMENTAL SCIENCES

ISSN 1001-0742
CN 11-2629/X

March 1, 2013 Volume 25 Number 3
www.jesc.ac.cn

PM10 PM2.5 OC EC PM2.1



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Chinese Academy of Sciences

ISSN 1001–0742 **Journal of Environmental Sciences** Vol. 25 No. 3 2013

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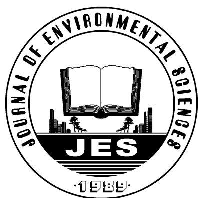

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ScienceDirect

Journal of Environmental Sciences 2013, 25(3) 613–625

JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Hydrocarbon biodegradation and dynamic laser speckle for detecting chemotactic responses at low bacterial concentration

Melina Nisenbaum[1,*], Gonzalo Hernán Sendra[2], Gastón Alfredo Cerdá Gilbert[3], Marcelo Scagliola[3], Jorge Froilán González[1], Silvia Elena Murialdo[1]

1. National University of Mar del Plata, Faculty of Engineering, Department of Chemistry, Biochemical Engineering Group. Juan B. Justo 4302, (7600), Mar del Plata, Argentina. E-mail: melinanisenbaum@gmail.com
2. National University of Mar del Plata, Faculty of Engineering, Laser Laboratory, 7600, Mar del Plata, Argentina and Optical Research Center, CONICET, La Plata-CIC. PO Box No. 3 1897, Gonnet, La Plata, Argentina
3. OSSE. Obras Sanitarias Mar del Plata - Batán S.E. Quality Management, Water Laboratory. French 6737, 7600, Mar del Plata, Argentina

Received 05 February 2012; revised 28 May 2012; accepted 03 July 2012

Abstract

We report on the biodegradation of pure hydrocarbons and chemotaxis towards these compounds by an isolated chlorophenol degrader, *Pseudomonas* strain H. The biochemical and phylogenetic analysis of the 16S rDNA sequence identified *Pseudomonas* strain H as having 99.56% similarity with *P. aeruginosa* PA01. This strain was able to degrade *n*-hexadecane, 1-undecene, 1-nonene, 1-decene, 1-dodecene and kerosene. It grew in the presence of 1-octene, while this hydrocarbons is toxic to other hydrocarbons degraders. *Pseudomonas* strain H was also chemotactic towards *n*-hexadecane, kerosene, 1-undecene and 1-dodecene. These results show that this *Pseudomonas* strain H is an attractive candidate for hydrocarbon-containing wastewater bioremediation in controlled environments. Since the classical standard techniques for detecting chemotaxis are not efficient at low bacterial concentrations, we demonstrate the use of the dynamic speckle laser method, which is simple and inexpensive, to confirm bacterial chemotaxis at low cell concentrations (less than 10^5 colony-forming unit per millilitre (CFU/mL)) when hydrocarbons are the attractants.

Key words: dynamic laser speckle; microorganisms; biodegradation; chemotaxis; hydrocarbons

DOI: 10.1016/S1001-0742(12)60020-5

Introduction

Bioremediation of contaminated sites is seen as one of the best remediation alternatives (Furukawa, 2003). Several bacteria that grow on hydrocarbons have been identified (Bogan et al., 2003; Shim et al., 2009). *Pseudomonas* is one of the most frequently-isolated bacterial genera capable of degrading polycyclic aromatic hydrocarbons (PAHs) (Zhao and Wong, 2009; Haritash and Kaushik, 2009). *Pseudomonas stutzeri* degrades PAHs compounds (Olivera et al., 2003), and some strains of *Pseudomonas aeruginosa* grow on a large number of hydrocarbons (Zhang et al., 2005; Obayori et al., 2009; Nie et al., 2010).

Several industrial wastewaters contain a toxic mixture of chlorophenols and hydrocarbon compounds (Finklea et al., 1998; Chrzanowski et al., 2011). Even though the ability to degrade hydrocarbons (Nie et al., 2010) and chlorophenols (Murialdo et al., 2003; Wolski et al., 2006; Durruty et al., 2011a, 2011b) has been found in several bacteria, few reports exist (Bruins et al., 2000) of isolated *Pseudomonas* able to catabolize both hydrocarbons or chlorophenols without the use of supplemental nutrients. Such a degrader could reduce considerably the cost of bioremediation.

The bioavailability of the pollutants and the bacterial distribution in a medium are among the major limitations for an efficient bioremediation (Semple et al., 2004). Additionally, properties such as motility, chemotaxis (Marx and Aitken, 2000), production of surfactants (Nie et al., 2010; Zhang et al., 2005; Maier and Soberón-Chávez, 2000), and the capability of using multiple substrates (Johnsen et al., 2005), play a critical role in biodegradation.

Bacterial chemotaxis is the direct movement of microorganisms in favor or in opposition to a gradient of substrate concentration, with the aim of finding ideal conditions for growth and survival (Paul et al., 2006). Several microorganisms chemotactic towards different pollutants have been isolated and characterized (Paul et al., 2005). Marx and Aitken (2000) demonstrated that lack of motility

* Corresponding author. E-mail: silviaem@fi.mdp.edu.ar, froilan@fi.mdp.edu.ar

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and chemotactic response towards naphthalene retards degradation. Different assays have been developed to show qualitative bacterial chemotaxis in semisolid and liquid mediums (Jin and Hereld, 2009; Murialdo et al., 2009). In all of them, spatial bacteria accumulation is observed at different times using white light. In these assays, low concentrations of bacteria can hinder the visualization of the chemotactic bacterial accumulation. We have recently developed a new method for the determination of chemotaxis, based on dynamic speckle activity segmentation in swim assays (Murialdo et al., 2009). This method allows differentiation of diverse degrees of motility in bacteria and has been tested with readily assimilable carbon sources, such as tryptone, to help the bacterial growth and facilitate the production of high concentrations of motile bacteria. However, bacterial growth on hydrocarbons as sole sources of carbon and energy leads to slow bacterial growth and low cell yields (Johnsen et al., 2002). The dynamic speckle laser method has not yet been tested with toxic compounds metabolizable by bacteria, where only a low concentration (less than 10^5 CFU/mL) of motile microorganisms occurs, which hinders the results of conventional chemotaxis visualization techniques.

Some recent reports on chemotaxis and hydrocarbon biodegradation by *Pseudomonas* are available (Wood, 2009), but no report exists so far on its behavior in *n*-hexadecane, 1-undecene, 1-nonene, 1-decene, 1-dodecene and kerosene mixtures. The exploration of safe and efficient methods to remove environmental pollutants has its major impetus in the search for novel biosurfactant-producing, chemotactic and multiple toxic compound-degrading microorganisms. Therefore, this research is focused on the biodegradation abilities and chemotactic responses of *Pseudomonas* strain H in the presence of some hydrocarbons which had not been previously studied with that strain. This strain was isolated from phenol-contaminated soil in Argentina, and is capable of using chlorophenols as its only carbon source (Murialdo et al., 2003; Wolski et al., 2006; Durruty et al., 2011a, 2011b).

Chemotaxis promotes biodegradation (Marx and Aitken, 2000), and the chemotactic response can go unnoticed using the conventional white light method when the concentration of bacteria is low. Therefore, the detection of chemotactic responses towards hydrocarbons when a low bacterial cell concentration is present (less than 10^5 CFU/mL), was tested with our recently reported method, using the dynamic speckle laser technique (Murialdo et al., 2009).

1 Experimental

1.1 Bacterial strains and growth media

The strain of *Pseudomonas* strain H used in this study was isolated from soil and was capable of degrading chlorophenols (Murialdo et al., 2003; Wolski et al., 2006; Durruty et al., 2011a, 2011b). Microorganisms were stored on agar plates at 4°C. The solid growth medium had the following composition: agar 1.5% (*W/V*) (Merck), LB 0.5% (*W/V*) (Luria Bertoni Broth, Difco), minimal growth medium (MS) and ampicillin 50 μg/mL. MS was prepared as described by Zhang et al. (2005) (g/L): $NaNO_3$ 4.0 (Merck), NaCl 1.0 (Timper), KCl 1.0, $CaCl_2 \cdot 2H_2O$ 0.1 (Merck), KH_2PO_4 3.0 and $K_2HPO_4 \cdot 12H_2O$ 3.0 (Sigma), $MgSO_4$ 0.2 (Anedra), and $FeSO_4 \cdot 7H_2O$ 0.001 (Merck).

The bacteria were grown in MS supplemented with 0.5% (*W/V*) LB and 0.001% (*W/V*) yeast extract as a vitamin source. These liquid cultures were incubated in the dark at 150 r/min and 20°C (environmental temperature of the isolation). Sterile flasks containing MS, 0.001% (*W/V*) yeast extract as a vitamin source and 0.5% (*V/V*) pure hydrocarbon mixture or glycerol (99%, Sigma, USA) as the only carbon source were inoculated with *Pseudomonas* strain H at the exponential growth phase, in order to induce hydrocarbon catabolic genes. The hydrocarbons used were the following (*V/V*): *n*-hexadecane 99%, 1-undecene 99%, 1-octene 97%, 1-nonene 99%, 1-decene 94%, 1-dodecene 95%, toluene 99% and kerosene 99% (Sigma), at final concentration of 0.5% (*V/V*). These flasks were called stock cultures. We considered the bacteria to be acclimated to the hydrocarbon mixture, when the extent of hydrocarbon degradation percent per unit time was the same after each hydrocarbon medium replenishment in the flasks. During acclimation, after 7 days, 90% of the liquid medium was discarded and replenished with sterile fresh medium that contained a mixture of hydrocarbons and MS medium adjusted to the initial values. The remaining 10% (*V/V*) (10^6 CFU/mL) served as inoculum. For all the assays a 10% (*V/V*) stock culture in the exponential phase of microbial growth was used. To have better air-liquid contact, all experiments were carried out in 250 mL flasks as batch reactors containing 80 mL of growth medium. All experiments were carried out in duplicate, with more than five repetitions.

1.2 Biomass growth

Biomass growth in the presence of hydrocarbons as the only carbon and energy source was determined by measuring cellular growth from hydrocarbon liquid cultures at OD_{600} with a UV-Visible spectrophotometer (UV1601PC, Shimadzu, Japan). 80 mL of minimal growth medium MS supplemented with 0.001% (*W/V*) yeast extract, 0.5% (*V/V*) single pure hydrocarbon and cellular concentration of 0.1 (OD_{600}) were incubated in 250 mL flasks at 150 r/min at 25°C. A positive control was prepared with LB and a negative control without any carbon source.

In these conditions, strain growth curves were expressed as $OD_{600}\ f(t)$. To model the growth parameters and determine the viable strain count, these data was converted into CFU $f(t)$ using a correlation between CFU as a function of OD_{600} as reported by other authors (Dalgaard and

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Koutsoumanis, 2001). To develop this correlation, during the exponential growth phase of each culture in liquid MS medium with hydrocarbon as the only carbon and energy source, 3 samples of the medium were taken at regular time intervals. For each sample, after having recorded the OD_{600}, enumeration of the bacteria (N_b, CFU/mL) was carried out on Petri dishes which contained MS supplemented with LB 2% (*W/V*), 0.5% (*V/V*) single pure hydrocarbon and agar-agar 2% (*W/V*) that were incubated at 20°C for 5 days. A regression line of the following form was obtained:

$$\log N_b = 0.590 \text{Abs}_{600\text{ nm}} + 8.562 \quad (1)$$

1.3 Hydrocarbon biodegradation

Five sterile 125 mL flasks were incubated with 40 mL of MS, 0.001% (*W/V*) yeast extract and 0.5% (*V/V*) of single pure hydrocarbon at 150 r/min at (20 ± 2)°C. The initial cellular concentration was 0.1 (OD_{600}) for all the assays. The total volume of culture in each flask was used for the hydrocarbons determination. The negative control had no bacterial inoculums and was incubated in the same conditions to test for possible loss of hydrocarbons by physical and chemical degradation and/or volatilization. The hydrocarbon concentration was measured by gas chromatography (GC) at 0, 24, 48, 72 and 96 hr. The negative control was measured at 96 hr. Every flask was covered with aluminum foil and sealed with adhesive tape to minimize evaporation. GC was conducted on a GC-17 gas chromatograph (Shimadzu, Japan), equipped with a Shimadzu AOC-17 split/splitless injection system, a Supelco SPB-5 column (30 m × 0.32 mm, film 0.50 μm) and a flame ionization detector (FID). Nitrogen of 5.0 grade was used as the carrier gas.

The hydrocarbons tested with the available column were as follows: *n*-hexadecane, 1-undecene, 1-octene, 1-nonene, 1-decene, 1-dodecene. The non-saponificable fraction from the flasks was obtained by extracting three times with 50 mL of hexane (Sintorgan, Argentina, pesticide grade) while shaking for 2 min. The extracts were dried with anhydrous sodium sulphate and the volume was reduced to 2 mL by rotary evaporation (BÜCHI R-114) at 70°C.

GC conditions to test for the presence of alkenes were the following: splitless mode at column rate 11.15 mL/min, column pressure of 207 kPa, linear velocity of 109.78 cm/sec and total rate of 135 mL/min. The column temperature was programmed from 40°C (2 min hold) to 300°C (2 min hold), with a temperature rise rate of 10°C/min. The temperature of the injection system was 250°C and the FID detector was 320°C. The total duration of the test was 30 min. GC conditions to test for the alkane *n*-hexadecane were the following: splitless mode at column rate 4.36 mL/min, column pressure of 116 kPa, linear velocity of 60.18 cm/sec and total rate of 226 mL/min. The column temperature was programmed from 60°C (3.5 min hold) to 300°C (14.50 min), rising at a rate of 7.5°C/min. The temperature of the injection system was 310°C and the FID detector was 320°C. The total length of the test was 50 min. The detector range was 1 for both GC conditions. The tests were repeated from three to five times.

To test PAH degradation, two sterile 125 mL flasks were incubated with 40 mL of MS, 0.001% (*W/V*) yeast extract and 0.5% (*V/V*) of kerosene in the same conditions as for the GC-tested samples. The initial cellular concentration was 0.1 (OD_{600}). The negative control had no bacterial inoculums. Extractions were the same as for the GC analysis. The flasks were measured by fluorescence at the beginning of the experiment and after 7 days of incubation. The aromatic fraction was measured on a Perkin Elmer LS55 fluorometer (excitation wavelength 310 nm, emission wavelength 360 nm, slit 15.0 nm).

1.4 Microorganism characterization

1.4.1 Microorganism identification

The isolates were checked for purity by streaking on plates with LB at 30°C, and stored in glycerol at 70°C. Phenotypic analyses (API 20 NE and API ZYM, BioMérieux, USA) were carried out according to the manufacturer's recommendations, to determine the characteristic profile for assimilation of organic compounds as sole carbon sources. The inocula were taken from cultures grown for 16–20 hr at 20°C on nutrient agar. Conventional phenotypic tests were done according to Barrow and Feltham (1993). The morphology of colonies was analyzed by direct observation, and cellular morphology and number and place of flagella by transmission electron microscopy at 75 kV (H-600AB, Hitachi, Japan). The cells were fixed with 2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4), and then washed in the same buffer followed by negative staining. Next, the sample was deposited on a copper grid containing a Formvar membrane, and after removing excess sample, the sample was dried. Finally, the preparation was stained with phosphotungstic acid (1%, pH 7.39) for one min.

The identification of fatty acids was previously made by Murialdo et al. (2003). The nearly full-length (1302 nucleotide positions) 16S rRNA gene of the strain employed in this study was amplified by Polymerase Chain Reaction (PCR), using the following primers from the laboratory of Microbiology, Biology Department, Balearic Islands University and the Mediterranean Institute of Advanced Studies (Palma de Mallorca, Spain): 16F357, 16R610 and 16F945, and sequenced as previously described by Gomila et al. (2005). The sequencing reactions were carried out using the ABI Prism Big Dye version 3.1 Terminator Cycle sequencing kit and the sequences were read with an automatic sequence analyzer (ABI Prism 3730 DNA Sequencer, Applied Biosystems). The sequences obtained were aligned with sequences from the GenBank/EMBL/DDBJ data base using a hierarchi-

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cal method for multiple alignments implemented in the Clustalx program (Thompson et al., 1997). The automatically aligned sequences were edited manually. Similarities and evolutionary distances were calculated with programs contained in PHYLIP (Phylogenetic Inference Package, version 3.5).

As part of the microbial characterization, we searched for genes involved in denitrification, and used primers to amplify *NirK, NirS* and *NosZ* genes (Robles Cortés, 2006). Bacterial motility was observed with an optical microscope (40×) under phase contrast, after 24 hr of incubation with tryptone. The thermal tolerance was determined, as part of the microbial characterization, by incubation of the strain in Petri dishes with LB 1.5% (*W/V*) on agar-agar at different temperatures. The presence of plasmids was analyzed through Boehringer Mannheim small-scale preparations of purified plasmid DNA (for the isolation of high purity plasmid (10 mg) as a standard protocol). The antibiotic resistance or susceptibility was tested by Luria Bertani plates (LB) supplemented with 30 μg/mL of antibiotic. The strains were inoculated onto the media and grown for 20–48 hr at 35°C.

1.4.2 Biosurfactant production

The *Pseudomonas* strain H isolated from soils contaminated with chlorophenols (Murialdo et al., 2003) was studied for the ability to produce biosurfactants. 2 mL of stock culture in its exponential phase was inoculated in batch cultures with 80 mL MS, 0.001% (*W/V*) yeast extract and 0.5% (*V/V*) of glycerol (99%, Merck), and incubated at 20°C in a shaker at 150 r/min for five days until the stationary phase was obtained. The cells were centrifuged at 3000 ×*g,* and the supernatant was used to determine the presence of biosurfactant using the drop-collapsing test (Jain et al., 1991). The emulsifying activity was estimated by adding 0.5 mL of *n*-hexadecane and 0.5 mL of culture to 4 mL of distilled water and shaking for 10 min. After standing one minute, the evidence of emulsification was then confirmed by the diminution of surface tension.

The colorimetric method proposed by Siegmung and Wagner (1991) was used to detect extra-cellular glycolipid production in the different cultures with pure hydrocarbons. 200 μL of the 24 hr hydrocarbons-growth batch cultures were inoculated into shallow wells in blue agar Petri dishes. The blue agar had the following composition: agar 1.5% (*W/V*), cetyltrimethylammonium bromide 0.2 mg/mL (CTAB, Sigma), methylene blue 5 μg/mL, dextrose 2% (*W/V*). The plates were incubated for 48 hr at 20°C and checked periodically. The biosurfactants were detected by the formation of dark blue halos with sharply defined edges around the cultures. This was caused by the presence of a precipitate formed by the cationic CTAB and the Methylene Blue in the presence of the anionic biosurfactant. In order to intensify the color of the halo, the plates were incubated at 4°C for two weeks.

1.5 Chemotactic assays

As a colony grows on a surface, it decreases the concentration of metabolizable attractants where the cell density is highest (Jin and Hereld, 2009). As a result, a spatial gradient is created, and the cells migrate outward in response to that gradient. Typically, the edge of the colony shows a sharp ring where the cells congregate in the steepest part of the gradient (Jin and Hereld, 2009). At low agar concentrations (0.25%–0.4% semisolid agar) the channels are sufficiently large that the bacteria can swim through them, and the assay is called a swim assay (Jin and Hereld, 2009). With some attractants, the colony expands equally throughout the agar. If the attractant can only be metabolized aerobically, the ring may only form on the surface (Jin and Hereld, 2009). Chemotactic rings can be documented photographically by using a "bucket of light" (Parkinson, 2007). We used a stock culture of *Pseudomonas* strain H in the exponential growth phase, which was centrifuged for 2 min at 2000 ×*g* to separate impurities. Then, cells were harvested by centrifugation at 3000 ×*g* for 20 min and resuspended in motility buffer (MB) (0.1 mmol/L EDTA and 10 mmol/L K_3PO_4, pH 7), previously vortexed to achieve good aeration. Tubes with bacteria were incubated at 150 r/min at 20°C with no carbon source for 24 hr before the chemotactic assays.

The swim assay was performed to determinate the chemotactic response towards different pure hydrocarbons. For this assay, 3 μL of the previous prepared cells were inoculated in the center of a Petri dish with agar 0.2% (*W/V*) in MB, triton-X 0.01% (*V/V*), and 0.025% (*W/V*) LB (positive control) or 0.25% (*V/V*) pure hydrocarbon. The negative control was carried out without any carbon source in the agar. The dishes were incubated at 20°C in a wet chamber and were examined every 24 hr. In the presence of a chemotactic response, a sharp chemotactic ring of bacteria grows and moves outwards from the inoculum and is visualized in the dish (Parales and Harwood, 2002). The chemotactic response is based on the created gradient due to the metabolization of the carbon source, and the consequent movement of the bacteria (Wolfe and Berg, 1989). Photographs were taken with a charge-coupled device (CCD) camera, using white light or a speckle laser as illumination sources. The conventional technique consists of observing the plate illuminated with white light from the bottom (Armstrong, 1967; Parkinson, 2007). The biospeckle method was implemented as described by Murialdo et al. (2009). An expanded and attenuated HeNe laser at wavelength 632.8 nm and 30 mW power illuminated the plate under study from the bottom through a ground glass diffuser. A CCD camera connected to a frame grabber registered a sequence of 8 bit images and 768 × 576 squared pixels, and stored it in the computer. A constant 4-Hz sampling frequency was used, and the camera integration exposure time was set at 40 msec. A pseudo-colored image was obtained after a processing

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stage, where the higher energy regions correspond to higher intensity bacterial movement (Sendra et al., 2005).

1.6 Kinetic parameter determination (Blanch and Clark, 1996)

The batch growth curve has 4 stages. As we used the same microorganism, the duration of each of these phases was a function of the composition (hydrocarbon) of the medium. There is a stage where microorganism growth occurs at a constant specific rate (μ) called the exponential phase. Data were taken at the end of this phase, since the maximum microbial concentration (X_f, CFU/mL) is then reached. Kinetics parameters (K_S, μ_{max}) were determined with the Monod equation:

$$r_x = \mu X \quad (2)$$

$$\mu = \frac{\mu_{max} S}{K_S + S} \quad (3)$$

where, S (mg/L) is the limiting substrate concentration (hydrocarbon), μ_{max} (hr^{-1}) is the maximum specific growth rate of the microorganism, X (CFU/mL) is the biomass concentration expressed as CFU and K_S is the saturation constant, which reflects the microbial affinity for the substrate.

Replacing Eq. (2) in Eq. (1):

$$r_x = \frac{\mu_{max} S}{K_S + S} \times X \quad (4)$$

At the beginning of the reaction all the nutrients are in excess, and in particular the concentration of the limiting substrate greatly exceeds K_S, so the former Eq. (3) reduces to (in exponential phase):

$$r_x = \frac{\partial X}{\partial t} = \mu_{max} X \quad (5)$$

Eq. (4) is integrated with $t = 0$ (time, hr), and $X = X_0$ (initial concentration of microorganisms) to:

$$\ln X = \ln X_0 + \mu_{max} t \quad (6)$$

We plotted ln$X$ vs. t (reaction time) to get μ_{max} (slope) in the exponential growth phase. Since the maximum specific growth rate (μ_{max}) is expressed per biomass unit (CFU), it generally provides specific information about the metabolic activity of the organism (or biomass) during cultivation.

From Eq. (5) we calculate the generation time (t_d, min) of the organism (time period in which biomass is doubled):

$$X = 2X_0 \quad (7)$$

Therefore,

$$t_d = \frac{\ln 2}{\mu_{max}} \quad (8)$$

If $t = t_t - t_L$, where t is equal to end time (t_t) minus lag phase (t_L), then Eq. (5) is rewritten:

$$\ln X = \ln X_0 + \mu_{max} \cdot (t_t - t_L) \quad (9)$$

Taking any value of X in the exponential phase (X_e), then t_L (duration of lag phase, hr) was calculated:

$$t_L = t_t - \frac{1}{\mu_{max}} \ln \frac{X_e}{X_0} \quad (10)$$

The correlation of A_{600} vs. biomass indicated that A_{600}=1 corresponded to 0.5 mg/L. Therefore, the global yield coefficient biomass-substrate ($Y_{X/S}$) was calculated as the slope of the plot: (X-X_0) vs (S-S_0):

$$(X - X_0) = Y_{X/S} \times (S_0 - S) \quad (11)$$

The hydrocarbon conversion percent was calculated as:

$$(S_0 - S) \times 100 / S_0 \quad (12)$$

2 Results

2.1 Microorganism identification

According to the fatty acid composition, the strain H had been reported as closely related to *P. aeruginosa* (Murialdo et al., 2003). In this study it was observed that the isolated bacteria were catalase and oxidase positive. It is a motile, rod-shaped (0.7 μm in diameter and 2 μm in length), Gram-negative bacterium with a single polar flagellum (**Fig. 1**).

Pseudomonas strain H used inositol, sorbitol, rhamnose, arabinose and melibiose and grew at temperatures ranging between 20°C–42°C. The strain was observed to be resistant to 30 μg/mL of amoxicillin, ampicillin, kanamycin, trimethoprim, bacitracin, cephamandole, cefuroxime vancomycin, or carbenicillin. It was also resistant to optochin. The bacteria were sensitive to amikacin, chloramphenicol, novobiocin, rifampin, sulfonamides, nalidixic acid, norflaxin, tetracyclines, gentamicin, streptomycin, azlociline, mezlocillin and colistin. The enzymatic characterization (API ZYM, BioMérieux, USA) revealed that the isolated *Pseudomonas* strain H has esterase (C4), phosphoamidase and lipase (C8) activities. According to biochemical analysis (API 20 NE, BioMérieux, USA) and phylogenetic analysis of the 16S rDNA sequence, *Pseudomonas* strain H was identified as *Pseudomonas aeruginosa* (99.56% similarity with *P. aeruginosa* PA01).

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A very specific detection, mostly at the strain level, could be achieved with genes involved in denitrification reductase (Robles Cortés et al., 2006). Nitrite reductase was not present in the bacteria, or the primers used did not amplify the genes involved, although nitrous oxidase reductase (*nosZ* genes) were amplified with a band size of 444 pb (pair bases) and SD = 5.37, according to the NCSA software program Gel Reader 2.05 (the National Center for Super-computing Applications, Champaign, Illinois, USA). The presence of one plasmid was also identified in the isolated strain through Boehringer Mannheim small-scale preparations.

2.2 Hydrocarbon degradation

The chlorophenol degrader *Pseudomonas* strain H isolated from contaminated soil (Murialdo et al., 2003), proved to be able of growing in hydrocarbons as the only carbon and energy source. Different flasks were inoculated with bacteria (OD_{600}= 0.1) and pure single hydrocarbons (0.5%, *V/V*) as the only carbon source (**Table 1**).

This strain was able of growing with 0.5% (*V/V*) *n*-hexadecane, 1-undecene, 1-nonene, 1-decene, 1-dodecene or kerosene as the sole source of carbon and energy, which was evident from the increase in cell concentration, and the disappearance of hydrocarbons from the flasks (**Table 1** and **Fig. 2**). Biomass was increased with incubation time in all cases. The flasks also exhibited formation of a visible biofilm in the air-water interface of the culture flasks (data not shown). The hydrocarbons flasks with *n*-hexadecane and kerosene showed the highest specific growth rate (μ, hr^{-1}) and bacterial growth percentages (calculated with respect to positive control with LB) (**Table 1**). The yield was higher in the presence of *n*-hexadecane. This hydrocarbons and kerosene presented the shortest lag phase and generation time among the studied hydrocarbons. Results with alpha olefins have been ranked in the following order (**Table 1**): specific growth rate, 1-decene = 1-nonene > 1-octene = 1-dodecene > 1-undecene. Yield, 1-undecene > 1-dodecene > 1-decene > 1-nonene > 1-octene. Lag phase, 1-undecene > 1-nonene > 1-dodecene > 1-octene > 1-decene. Generation time, 1-nonene > 1-decene > 1-dodecene > 1-octene > 1-undecene. Bacteria with toluene as the only carbon source did not show cell growth or biofilm formation in the flask (data not shown). Preliminary assays with cumene, hexane, premium grade diesel fuel and unleaded gasoline as the only carbon source in MS showed considerable cellular growth as determined by turbidity in the flasks, compared with negative control without bacteria.

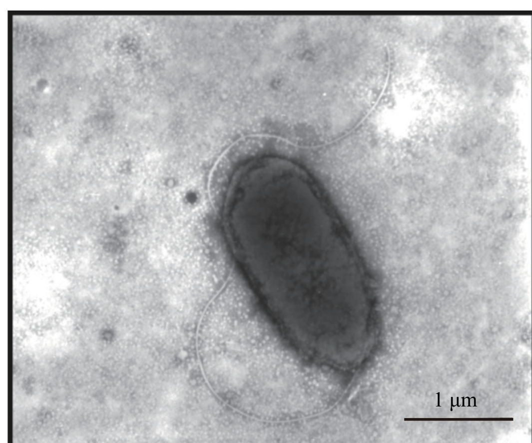

Fig. 1 Transmission electron micrographs of negative stained cell of *Pseudomonas* strain H. The observation was made in a transmission electron microscope, Hitachi H-600AB at 75μV.

The hydrocarbon degradation patterns determined by GC are shown in **Table 1**, and percent of hydrocarbons conversion during the assay in **Fig. 3**. The compounds *n*-hexadecane, 1-undecene, 1-decene, 1-dodecene and 1-nonene were degraded over 50% (*W*/*V*) of the initial concentration. All the flasks had physical and/or chemical loss of hydrocarbons between 6.86% and 14% of hydrocarbon after 96 hr of incubation, except that *n*-hexadecane that did not show any loss in the negative control. The degree of physical loss was attributed to the low molecular weight of the compounds, as the heavier ones exhibited less evaporation than the lighter ones in the negative controls. The highest degradation was achieved with *n*-hexadecane.

Table 1 Hydrocarbon biodegradation and cell growth, using hydrocarbon as the only carbon source

Sample	Cellular growth* (%)	Initial hydrocarbon concentration (mg/L)	Final hydrocarbon concentration (mg/L)	Biodegradation (%)**	Physical or chemical loss*** (%)	Specific growth rate μ (hr^{-1})****	$Y_{X/S}$	t_L (hr)	t_d (hr)
LB	100.00	–	–	–	–	0.075	–	1.65	9.24
n-Hexadecane	46.71	3655.33±0.01	503.32±0.01	86.23	0	0.015	9.59×10^{-5}	0	46.83
1-Undecene	22.63	3717.12±0.03	1920.03±0.03	55.64	13.11	0.003	9.05×10^{-5}	0.206	203.87
1-Decene	11.68	3489.20±0.02	650.88±0.01	74.49	6.86	0.007	4.79×10^{-5}	78.91	101.93
1-Octene	5.11	3645.45±0.02	0	?	?	0.006	1.55×10^{-5}	24.27	126.03
1-Nonene	21.17	3601.72±0.01	0	87.41	12.59	0.007	3.27×10^{-5}	15.52	93.67
1-Dodecene	18.25	3785.70±0.03	1783.27±0.02	61.81	14.43	0.006	7.07×10^{-5}	15.81	115.52
Kerosene	34.30	7.67±0.03	7.72±0.03	–	–	0.0117	–	4.67	59.24

All the values were calculated by subtracting the negative control; values for hydrocarbon concentration in kerosene (gray highlighted) correspond to PAH determination by fluorescence.

* Calculated with regard to the positive control as 100%; ** difference between final hydrocarbon concentration in flask with bacteria and the negative control (the hydrocarbons content in the negative control at the end of the assay was taken as the 100%); ***difference between initial hydrocarbon concentration (taken as 100%) and final hydrocarbon concentration in negative control. ****: linear regression from 0.916 to 0.988 in all assays.

–: unable to determine.

?: due to the high volatility of 1-octene it was not possible to determine the physical loss and subsequently the biodegradation capability.

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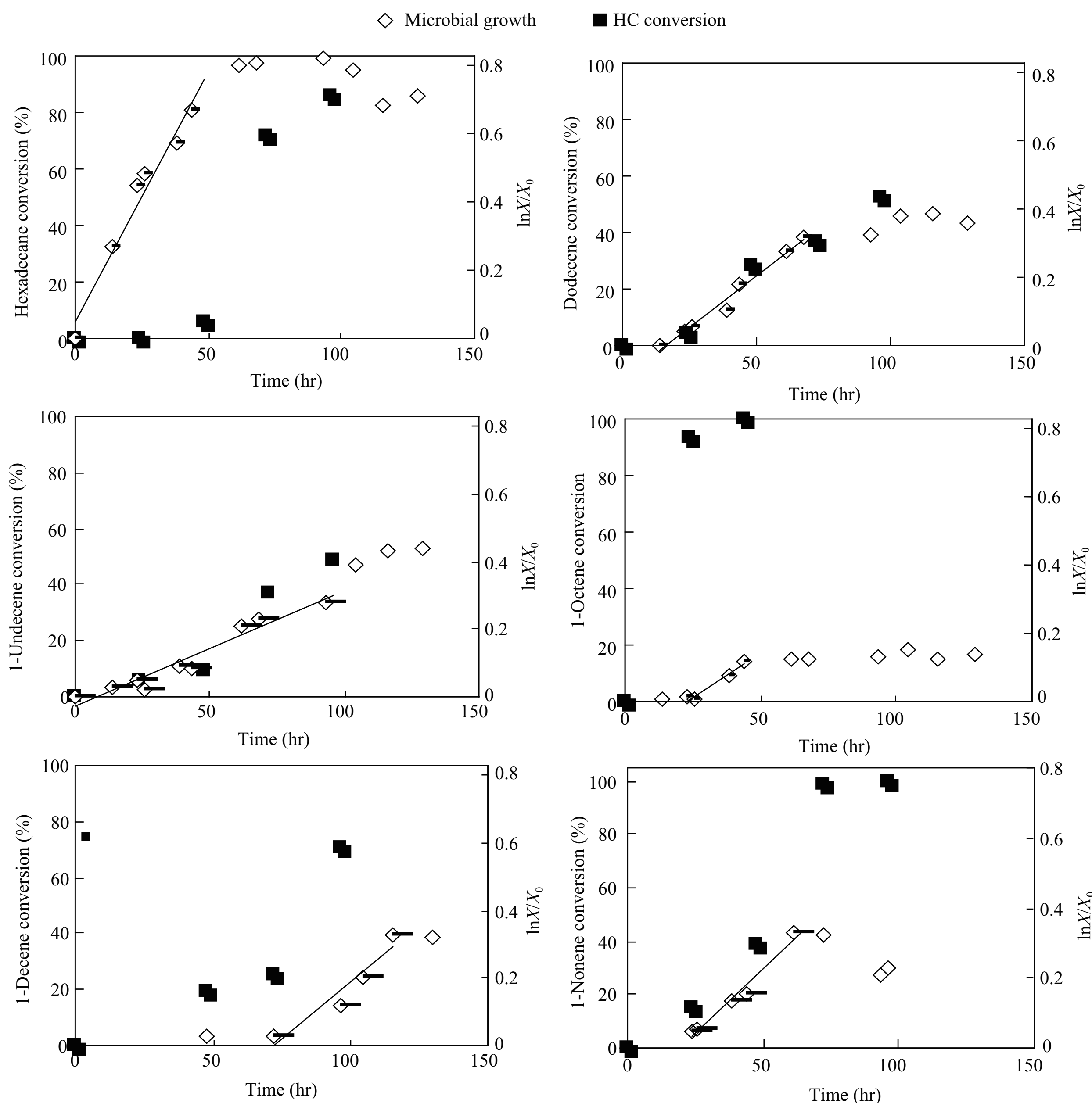

Fig. 2 Hydrocarbon degradation and microbial growth in the presence of hydrocarbons as the only carbon source. LB (Luria Bertani plates) was used as positive control. The slope of the straight line indicates the specific growth rate (μ) (Eq. (5)).

Even though *Pseudomonas* sp. was able to grow at the expense of 1-octene, the complete hydrocarbons-removal in the culture after 48 hr of incubation was not attributed to the bacterial degradation but to the high volatility of this compound (International Cards of Chemical Security, ICSC: 0934). A similar pattern was seen with 1-nonene, although the evaporation loss was less than with 1-octene and the bacterial growth was higher. The other hydrocarbons have been ranked in the following order of decreasing biodegradability: 1-decene, 1-undecene, 1-dodecene.

Polyaromatic hydrocarbons from batch cultures with kerosene as the only carbon source were measured by fluorescence at 0 and 7 days of incubation to test PAH degradation. The values indicated no degradation of the PAHs from kerosene, though the flask presented a high degree of turbidity with 34.30% cellular growth (**Table 1** and **Fig. 2**).

2.3 Extracellular presence of surfactants

A drop-collapsing test was used to test the presence of biosurfactants in the medium as part of strain characterization. A turbid stable emulsification in the flask with hexadecane-water indicated a decrease in the surface tension generated by surfactant compounds present in the medium, which shows that this strain has the ability to produce extracellular surfactants.

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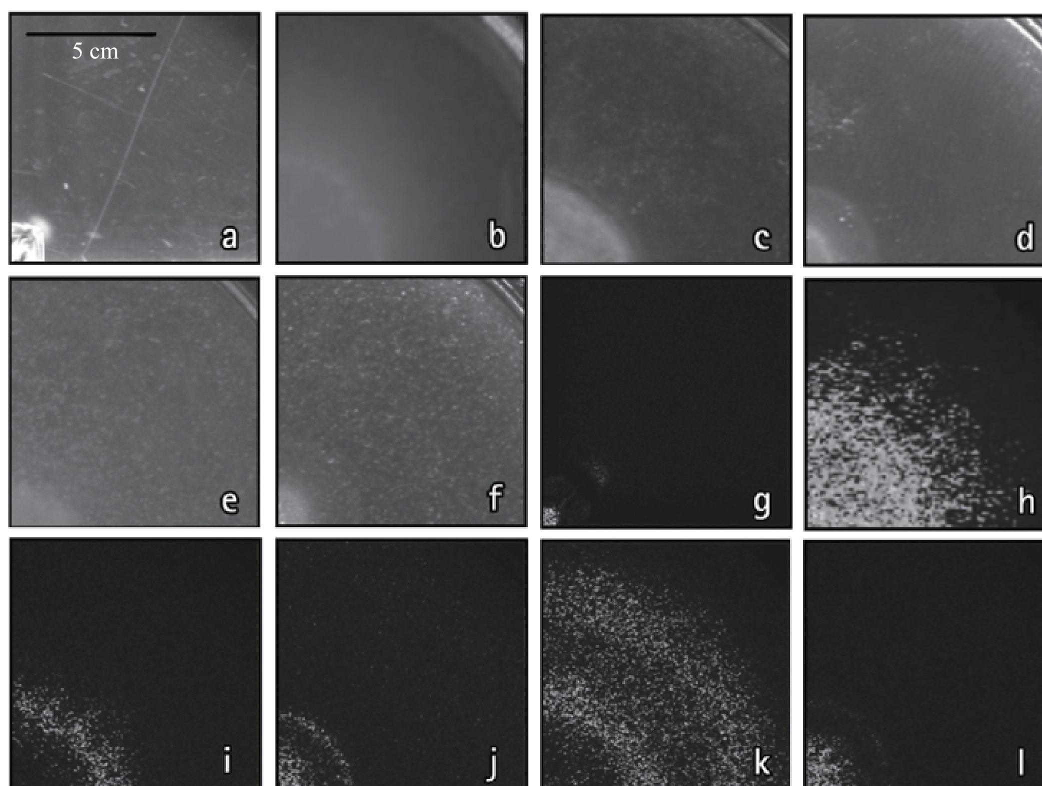

Fig. 3 Chemotactic responses of *Pseudomonas* strain H in the swim assay method detected with white light (a–e) and dynamic speckle laser (f–g). All dishes contained agar in motility buffer (a, g, negative control) and LB (b, h, positive control), *n*-hexadecane (c, i), 1-undecene (d, j), 1-dodecene (e, k), kerosene (f, l).

The colorimetric method used for a culture with glycerol exhibited intense blue halos in the presence of CTAB. The halos for the cultures with *n*-hexadecane, kerosene, 1-nonene, 1-dodecene, 1-decene and 1-undecene were light blue, possibly related to the presence of a lower concentration of surfactant in the medium. The presence of the halos indicated that this strain was capable of producing biosurfactants in the presence of hydrocarbons as the only source of carbon and energy.

2.4 Chemotactic responses

The chemotactic response of *Pseudomonas* strain H toward pure hydrocarbons was analyzed with swim assays. Chemotaxis was observed qualitatively as the formation of expanding rings of growth in response to chemical gradients created on semi-solid agar plates. Triton-X 0.01% (*V/V*) was used in the agar for better dissolution of the hydrocarbons. A chemotactic ring in the positive control was detected at 24 hr from the beginning of the assay, but the responses towards hydrocarbons were seen around 48 and 72 hr from the start of incubation. No bacterial response towards triton X-100 was found in a previous assay without hydrocarbon, as negative control. For this conventional technique, photographs were taken with white light and chemotactic responses were observed in the dishes with *n*-hexadecane, 1-undecene, 1-dodecene, and kerosene (**Fig. 3 c–f**).

No response was observed towards 1-decene and 1-nonene (data not shown). For the assay with 1-octene only a very weak response was detected, which was impossible to capture with the camera, of weakly consistent replicated data (data not shown). The 1-octene hydrocarbon is a very volatile compound and this feature may interfere with its use as an attractant in this assay. Some negative control plates showed weak chemotactic rings (**Fig. 3a**), probably due to some attractants in the agar, even though it was of the best purity available. Some of the swim assays using white light were not reproducible in all cases, given the fact that in some replicates no ring was visible to the human eye. The detection of chemotaxis using white light is therefore not an accurate method when the concentration of bacteria is low, as is the case when assaying with toxic compounds, and it is difficult to distinguish these results (**Fig. 3e**) from the negative control assays (**Fig. 3a**). The dynamic speckle laser was used not only to confirm the results obtained by the swim technique with white light, but also to evaluate, for the first time, its applicability in the case of chemotaxis to non-polar contaminants. The swim assays tested with the laser dynamic speckle technique used the following attractants: *n*-hexadecane, 1-undecene, 1-dodecene and kerosene (**Fig. 3** right column: i–l). All the laser speckle images showed more details than the photographs taken with white light. The speckle images (**Fig. 3k**) allowed us to observe two chemotactic rings not

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visible with white light (**Fig. 3e**). This may be related to the low density of mobile bacteria present in the area external from the inoculation. Also, the dynamic speckle laser image for 1-dodecene showed a remarkable intensity for the rings in the chemotactic area (**Fig. 3k**). This is due to the higher amount of mobile bacteria in the ring compared to the rest of the colony, allowing a discrimination of motility levels inside the colony. On the other hand, the white light images (**Fig. 3e, c**) showed a high density spot at the source of inoculation, which was not visible via dynamic speckle laser (**Fig. 3k, i**). This indicates that there were non-motile or dead bacteria. Also, images taken with white light in Fig. 3c show a white plume spread from the point of the inoculation, showing a high density of bacteria. This dynamic speckle laser image confirms that this was a zone with clusters of non-motile or dead bacteria (**Fig. 3i**), and the motile ones were in the external ring not visible with white light (**Fig. 3c**). Microorganism plate counts in areas where the white light did not show rings that were detectable by dynamic speckle laser, indicated less than 10^5 CFU/mL.

The results of the white light negative control without attractant (**Fig. 3a**) were confirmed with dynamic speckle laser (**Fig. 3g**), where no movement was detected. LB (positive control) was an attractant for the microorganism, as we see in the white light image (**Fig. 3b**), but the dynamic speckle laser method proved effective in showing bacterial motility beyond the external ring. This zone was not seen with white light due to the low bacterial concentration. Some images showed that the density of bacteria seen with white light (**Fig. 3d, f**) correlate well with motile bacteria seen with biospeckle (**Fig. 3j, i**). This indicates that both techniques are complementary in discriminating which part of the population is motile and alive, and which is sessile or dead.

3 Discussion

In nature there exist diverse microbial species that are capable of growing using toxic compounds as a food source (Paul et al., 2005). The use of ancillary carbon sources in addition to hydrocarbons may favor the growth of tolerant but not degrading microorganisms present in hydrocarbon-containing wastewater. A significant reduction in the cost of hydrocarbon wastewater treatment may be achieved by discovering new microorganisms able to degrade high concentrations of hydrocarbons without the addition of easily degradable carbon sources.

Hydrocarbon biodegradation rates have been shown to be highest for saturated hydrocarbons, followed by the light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting extremely low rates of degradation (Leahy and Colwell, 1990; Blanchard, 1966; Das and Chandran, 2011). *Pseudomonas* strain H isolated in this work agrees with those results, showing better biodegradation with the alkane *n*-hexadecane than with the other tested hydrocarbons. In the aerobic degradation of hydrocarbons, the initial intracellular attack of organic pollutants is an oxidative process, and the activation as well as incorporation of oxygen is the key enzymatic reaction catalyzed by oxygenases and peroxidase, converting organic pollutants step by step into intermediates of the central intermediary metabolism (Das and Chandran, 2011). Blanchard (1996) found that octene unsaturated at the 1 or 2 position kills microorganisms that grow on fuel. Our results showed that the bacterial growth was limited during the incubation time with 1-octene, which may be due to the evaporation of this compound, even though bacteria were able to grow at expense of this hydrocarbons. Blanchard (1966) also mentioned that the lethal effects of these short-chain olefins end with nonene, 1-decene and 1-dodecene. The *Pseudomonas* strain H investigated in this work, mostly related to *P. aeruginosa* PAO1, grew on all of these hydrocarbon compounds, showing that this strain might be an efficient hydrocarbon degrader. When using LB as positive control, we saw a slightly longer lag phase than with *n*-hexadecane and 1-undecene (**Table 1** and **Fig. 2**), maybe because the strain was previously grown on a mixture of the tested hydrocarbons stock solution.

Some authors (Mueller et al., 1991) have reported microorganisms able to degrade PHAs but without the ability to degrade PCP. Others (Chrzanowski et al., 2011) needed the addition of surfactants to reduce the toxicity of 4-CP and 2,4-DCP to the hydrocarbon-degrading cells.

The reported isolated strain was also capable of metabolizing chlorophenols (Wolski et al., 2006; Durruty et al., 2011a, 2011b) without the production of toxic byproducts (Ayude et al., 2009). In preliminary assays, we have also observed the growth of this strain in liquid mediums with cumene, hexanes, unleaded gasoline, and premium grade diesel fuel as the only carbon and energy source. Future assays of the behavior of *Pseudomonas* strain H in the simultaneous presence of hydrocarbons and chlorophenol mixtures will be of great importance, since hydrocarbon-rich wastewaters contain a great variety of often toxic co-contaminants. Pentachlorophenol (PCP), used as a wood preserving agent, was formulated as a 5% petroleum mixture (Chrzanowski et al., 2011).

Although *Pseudomonas* has been identified as one of the most frequently-isolated bacterial genera capable of degrading PAHs (Zhao and Wong, 2009; Haritash and Kaushik, 2009; Nie et al., 2010), in this work we could not find any degradation of PAHs present in kerosene during 7 days of incubation. As kerosene's composition is specified as 15.9% aromatics, 52.8% cycloparaffins, 30.8% paraffins, 0.5% alkenes (CAS No: 8008-20-6), the growth of *Pseudomonas aeruginosa* strain H in this medium could be due to the presence of linear compounds in the kerosene which are more easily degraded. Das and Mukherjee (2007), found in their research that all the microbes

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exhibited higher biodegradation capacity towards the *n*-alkanes fraction toward aromatics hydrocarbonss. Leahy and Colwell (1990) reported works which describe co-oxidation, in which non-growth hydrocarbons are oxidized in the presence of hydrocarbons which can serve as growth substrates, such as alkanes. More studies focused on PAHs with *Pseudomonas* strain H would be of great interest.

The bioavailability of hydrophobic organic pollutants for the degrading bacteria is a limitation for the efficient bioremediation of contaminated sites. This problem can be overcome using surfactants that facilitate direct contact between the microbes and non-polar toxic compounds (Paul et al., 2005). Biosurfactants increase the oil surface area and the amount of oil that is actually available for bacteria to utilize (Das and Chandran, 2011). We demonstrated that *Pseudomonas* strain H was capable of producing glycolipids, probably ramnolipids, which are typical surfactants of *P. aeruginosa* (Maier and Soberón-Chávez, 2000; Zhang et al., 2005; Nie et al., 2010). Zhang et al. (2005) demonstrated that both cell growth and biodegradation of crude oil were observed with addition of small amounts of either glycerol or rhamnolipid. This was closely associated with the facilitated biodegradation of crude oil caused by supplemented or accumulated rhamnolipids. In further studies, it would be interesting to characterize the glycolipids involved and the factors that increase the biosurfactant production in the medium, as well as the metabolic pathways.

When observing *Pseudomonas* strain H under the microscope, constant movement and higher activity was seen in media with hydrocarbons as the only carbon and energy source, in contrast with the medium with LB or glycerol. Due to the hydrocarbon toxicity, it is possible that these compounds generate in the microorganisms a situation of stress, and consequently modify their activity. Heipieper et al. (1994) have described lipophilic molecules responsible for the alteration on the structure and fluency of the cell membrane when these molecules are attached to the membrane. In this work, we observed that the level of motility depends on the substrate available for cell growth. It is possible that conditions of low soluble or low available carbon sources activate the membrane chemoreceptors involved in the chemotactic response towards metabolizable substrates.

Some authors attribute to the bacterial chemotaxis towards toxic substances an important role in the fate of pollutants in the environment (Parales and Harwood, 2002). The chemotaxis towards hydrocarbons has already been studied in bacteria able to degrade naphthalene (Grimm and Harwood, 1997), and BTEX compounds (Parales et al., 2001.). In addition, chemotaxis of an isolated hydrocarbon-degrading bacterium towards a pure alkane has also been reported (Lanfranconi et al., 2003). Some chemotactic Pseudomonads have been described with more hydrophobic pollutants, such as high-molecular-mass PAHs (Ortega-Calvo et al., 2003). Van Beilen et al. (2001) mention preliminary evidence of a chemotactic response of *Pseudomonas aeruginosa* towards hexadecane. In this study we have demonstrated for the first time chemotaxis in *Pseudomonas* strain H, closely related to *Pseudomonas aeruginosa* PAO1, towards *n*-hexadecane, 1-dodecene, 1-undecene and kerosene.

In the system of chemotaxis toward toluene for *P. putida* F1, the same trans-regulatory elements are required for induction of chemotaxis and degradation (Parales and Hardwood, 2002). *Pseudomonas* strain H did not show chemotactic responses to some compounds that could be used for growth by this strain as the sole carbon source. We can conclude that chemotaxis is not related with respect to the biodegradation of 1-nonene and 1-decene by *Pseudomonas* strain H. It would be interesting to investigate what role chemotaxis plays in the rate of mineralization of the studied hydrocarbons by *Pseudomonas* strain H.

Different assays have been developed to show qualitative bacterial chemotaxis in semisolid and liquid mediums, such as agarose plug assay, swarm or swim assay, drop assay, and capillary assay (Takayama et al., 1998; Mazumder et al., 1999; Jin and Hereld, 2009; Murialdo et al., 2009). In all of them, the chemotactic response is inferred by observing the spatial bacteria accumulation at different times using white light. In these assays, a low concentration of bacteria can hamper or preclude the visualization of the chemotactic ring, as occurred several times in this project while investigating chemotaxis towards hydrocarbons. The size, thickness, relative position, and depth of each ring provide important information about a strain's motility and tactic behavior (Parkinson, 2007). The chemotactic analysis by speckle laser recently presented by Murialdo et al. (2009), was applied in this work for the first time to investigate chemotaxis towards non polar pollutants, and facilitated an improved visualization of the ring at low concentration of bacteria. It can be used as a complement to the conventional white light images, not only to confirm chemotaxis towards hydrocarbons, but also to detect different degrees of motility in bacteria swimming plates. It also has higher definition for the detection of bacteria accumulation, as it identifies motility clusters. Furthermore, chemotactic analysis with dynamic speckle laser has the potential to further explore this phenomenon with other contaminants.

Traditional chemotaxis methods such as swim, swarm and drop assays use supports like agarose, agar-agar, alginates and other gelling agents. Having a reliable negative control with these materials is not always feasible since most bacteria could be attracted by these media, which can be degraded by the presence of agarase (Malmqvist, 1978; Fu and Kim, 2010). Weak chemotactic rings were visualized in some negative control dishes in this work. This could be due to a possible chemotactic attraction of the microorganisms towards the agar, but this was

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differentiated from the tested negative assays with the dynamic laser speckle method.

Biodegradation of hydrocarbons by *P. aeruginosa* has been studied (Das and Mukherjee, 2007), and there are some reports on chemotactic responses of *Pseudomonas* species to hydrocarbons (Ortega-Calvo et al., 2003). In addition, chemotaxis of an isolated hydrocarbon-degrading bacterium towards a pure alkane has also been reported (Lanfranconi et al., 2003). However, there are no previous investigations concerning biodegradation of hydrocarbons and chemotaxis of *P. aeruginosa* toward the compounds studied in this work (*n*-hexadecane, 1-undecene, 1-octene, 1-nonene, 1-decene, 1-dodecene and kerosene mixtures). The fact that *Pseudomonas* strain H can degrade chlorophenols (Murialdo et al., 2003; Wolski et al., 2006; Durruty et al., 2011a, 2011b) and all the tested hydrocarbons makes it an attractive candidate for hazardous wastewater bioremediation in controlled reactors. Since *Pseudomonas* strain H is closely related to *Pseudomonas aeruginosa* PAO1, its probable pathogenic properties may limit its use in non-enclosed environments. Some genetically modified microorganisms are being studied with success in laboratory-scale bioremediation processes (Parales and Harwood, 2002). Another possible alternative consists of transferring the catabolic plasmids to nonpathogenic microorganisms, with pre-existing high reproductive rate, and low nutritional requirements. This might enhance the degradation of toxic compounds by microorganisms that are part of the wastewater treatment plants' microflora. More research is needed to determine whether plasmids and genes from *Pseudomonas* strain H can be transferred to other microorganisms and whether these microorganisms can survive in mixed microbial and heavily contaminated natural environments. The metabolic pathways of chorophenol and hydrocarbon degradation by *Pseudomonas* strain H are still unknown. The capability to aerobically degrade aromatics is widely distributed among different bacterial taxa (Junca-Díaz, 2004). Metabolic pathways and encoding genes have been reported and characterized in many bacterial strains, predominantly belonging to Gram negative bacteria of the genera *Pseudomonas*, *Burkholderia*, *Ralstonia*, *Sphingomonas*, *Comamonas*, and *Alcaligenes*, and to Gram positive bacteria of the genera *Rhodococcus* and *Mycobacterium*. Under aerobic conditions, aromatic compounds are prepared for ring-cleavage by the introduction of hydroxyl functions, usually in *ortho*-position to one another (Junca-Díaz, 2004). Thus, the degradation of a broad range of aromatics proceeds via a few central intermediates such as catechol, gentisate, protocatechuate and hydroxyhydroquinone. The genes encoding the enzymes for aromatic degradation are commonly found in operons and often located on plasmids. Toluene degradation initiated by dioxygenation has best been studied in *Pseudomonas putida* F1 (Junca-Díaz, 2004). Activation of toluene by monooxygenation has been described in the *o*-, *m*-, and *p*-position. One of the best-studied systems for the degradation of methylsubstituted phenols is *Pseudomonas* sp. CF600 (Junca-Díaz, 2004).

Further research could provide new insights into the capacity of this microorganism to degrade other xenobiotic compounds and the nature of these genetic mechanisms. It was therefore of great importance to study the hydrocarbon degradative capacity, chemotactic responses and production of biosurfactants of this native chlorophenol degrader strain for application in bioremediation techniques.

4 Conclusions

We have shown that this chlorophenol degrader, called *Pseudomonas* strain H, is closely related to *Pseudomonas aeruginosa* PA01. This strain was able to degrade *n*-hexadecane, 1-undecene, 1-nonene, 1-decene, 1-dodecene and kerosene. This strain was also able to grow at the expense of 1-octene, although this hydrocarbons is toxic to other hydrocarbon degraders. It was also chemotactic toward *n*-hexadecane, kerosene, 1-undecene and 1-dodecene. Given the capability of this strain to degrade chlorophenols (Wolski et al., 2006; Durruty et al., 2011a, 2011b) and also hydrocarbons (this work), further research should be addressed towards investigating the co-degradation of both kinds of substrates by this *P. aeruginosa* PA01. Durruty et al. (2011a, 2011b) have shown that in several cases the simultaneous use of two toxic substrates by this strain improves overall performance. Due to its capacity to produce biosurfactants while degrading contaminants, this *Pseudomonas* strain H is a microorganism worth considering for the biodegradation of toxic compounds.

At the very beginning of the batch growth of microorganisms, the cell concentration is low. The early detection of the first chemotactic bacteria in a process of remediation is very important, because the necessary steps can be taken to facilitate chemotactic bacterial development. The present study has shown that the dynamic laser speckle technique can also be used to detect different degrees of motility and chemotaxis responses to hydrocarbons at low cell density. The method proposed here presents advantages over the conventional white light technique of chemotaxis detection by optical density, because the speckle technique allows distinguishing between different levels of motility, and can be used when densities of bacteria are low, two features not available with the conventional approach with white light.

Acknowledgments

We thank National University of Mar del Plata, CIOP (La Plata University), and OSSE (Obras Sanitarias Sociedad de Estado) where this research took place. We also thank CIC and CONICET for the fellowships, grants and salaries, and UIB (Islas Baleares University, Mallorca, Spain) where the

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molecular assays took place. S. Murialdo is a researcher of CIC. This work was supported by 15/G305 ING311/11 and 1535/10CIC.

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Journal of Environmental Sciences (Established in 1989)
Vol. 25 No. 3 2013

Supervised by	Chinese Academy of Sciences	**Published by**	Science Press, Beijing, China
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Edited by	Editorial Office of Journal of	Domestic	Science Press, 16 Donghuangchenggen
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ISSN 1001-0742

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