Kerosene tolerance in *Achromobacter* and *Pseudomonas* species

Mihaela Marilena Stancu

**Abstract**

**Purpose:** The aim of the present study was to investigate the tolerance of five new *Achromobacter* and *Pseudomonas* strains to kerosene and to establish if the production of several secondary metabolites increases or not when these bacteria were grown in the presence of kerosene. The biodegradation of kerosene by isolated bacteria was also investigated in this study.

**Methods:** Five Proteobacteria were isolated from different samples polluted with petroleum and petroleum products. Based on their morphological, biochemical, and molecular characteristics, isolated bacteria were identified as *Achromobacter spanius* IBBPo18 and IBBPo21, *Pseudomonas putida* IBBPo19, and *Pseudomonas aeruginosa* IBBPo20 and IBBPo22.

**Results:** All these bacteria were able to tolerate and degrade kerosene. Higher tolerance to kerosene and degradation rates were observed for *P. aeruginosa* IBBPo20 and IBBPo22, compared with that observed for *A. spanius* IBBPo18 and IBBPo21, and *P. putida* IBBPo19. All these bacteria were able to produce several secondary metabolites, such as surfactants and pigments. Glycolipid surfactants produced by *P. aeruginosa* IBBPo20 and IBBPo22, *A. spanius* IBBPo18 and IBBPo21, and *P. putida* IBBPo19 have a very good emulsification activity, and their activity increased when they were grown in the presence of kerosene. The production of rhamnolipid surfactants by *P. aeruginosa* IBBPo20 and IBBPo22 was confirmed by detection of *rhlAB* gene involved in their biosynthesis. Pyocyanin and pyoverdin pigments were produced only by *P. aeruginosa* IBBPo20 and IBBPo22, while carotenoid pigments were produced by all the isolated bacteria. Significant changes in pigments production were observed when *P. aeruginosa* IBBPo20 and IBBPo22, *A. spanius* IBBPo18 and IBBPo21, and *P. putida* IBBPo19 were grown in the presence of kerosene.

**Conclusion:** Due to their ability to tolerate and degrade kerosene, and also to produce several secondary metabolites, the isolated bacteria could be used in the bioremediation of kerosene-polluted environments.

**Keywords:** *Achromobacter*, *Pseudomonas*, Kerosene, Tolerance, Secondary metabolites

**Introduction**

The *Achromobacter* and *Pseudomonas* are two of the most diverse and ubiquitous Gram-negative bacterial genera whose species (sp.) were isolated worldwide from all types of environments, including from water, sediments, soil, and sludge (Leahy and Colwell 1990; Chikere et al. 2011; Mnif et al. 2011; Varjani 2017). Bacterial sp. of genera *Achromobacter* and *Pseudomonas* have considerable technological importance and include a variety of metabolically versatile bacteria with ability to utilize a wide range of simple and complex toxic organic compounds as the carbon and energy sources (Chikere et al. 2011; Varjani 2017). These Gram-negative bacteria produce large amount of secondary metabolites, such as surfactants (Abdel-Mawgoud et al. 2009; Rocha et al. 2011; Rikalović et al. 2015; Joy et al. 2017) and pigments (Malik et al. 2012; El-Fouly et al. 2015) which are essential for their survival and enable some species to tolerate and degrade organic compounds that enters into the environment during petroleum extraction, transportation, storage, and processing activities (Leahy and Colwell 1990; Rocha et al. 2011). Generally, aliphatic and aromatic hydrocarbons which exist in the composition of petroleum and petroleum products are very toxic for most of the bacteria. Nevertheless, a number of bacteria able to tolerate and
Bacteria used in this study were isolated from different environments of bacteria with high hydrocarbon degradative ability can serve as promising tools for the bioremediation of petroleum and petroleum product-polluted sites. The indigenous bacteria which were exposed to aliphatic and aromatic hydrocarbons become adapted, exhibiting induction and repression of specific enzymes, genetic modifications which result in new metabolic capabilities, and selective enrichment of bacteria able to tolerate and degrade the hydrocarbons (Leahy and Colwell 1990; Chikere et al. 2011; Matilla 2018). Consequently, the isolation from the contaminated environments of bacteria with high hydrocarbon degradability can serve as promising tool for the bioremediation of petroleum and petroleum product-polluted sites (Patowary et al. 2016). Kerosene, also known as paraffin or paraffin oil, is a refined petroleum product which contains up to 260 aliphatic and aromatic hydrocarbons (C6-C17), including toxic compounds, such as benzene, toluene, trimethylbenzene, ethylbenzene, xylene, n-hexane, trimethylpentane, naphthalenes, and other PAHs (Ritchie et al. 2003; Shahzadi et al. 2019). Many isolated bacteria possess the ability to completely degrade only simple petroleum hydrocarbons. It is generally recognized that no single species is able to completely degrade an complex mixture of hydrocarbons (Chikere et al. 2011; Patowary et al. 2016). However, several *Achromobacter* and *Pseudomonas* strains able to grow on kerosene as the carbon and energy source have been reported (Silva et al. 2006; Ahamed et al. 2010; Mazumdar et al. 2015).

The aim of the present study was to investigate the tolerance of five new *Achromobacter* and *Pseudomonas* strains to kerosene and to establish if the production of several secondary metabolites (i.e., surfactants, pigments) increase or not when these bacteria were cultivated in liquid medium overlaid with kerosene. The presence of *rhlAB* (rhamnosyl transferase) gene was investigated for the isolated bacteria grown under the same conditions. The biodegradation of kerosene by *Achromobacter* and *Pseudomonas* isolates was also investigated in this study.

**Materials and methods**

**Isolation and characterization of *Achromobacter* and *Pseudomonas* strains**

Bacteria used in this study were isolated from different water, soil, and oily sludge samples polluted with petroleum and petroleum products (Poeni, Teleorman County, Romania), through enrichment cultures method as previously described (Stancu and Grifoll 2011). Enrichment cultures were speeded on nutrient-rich LB agar (Sambrook and Russel 2001), and growing colonies were further purified by repeated streaking. The isolates were maintained routinely by subculture in liquid LB medium containing 20 μg ml⁻¹ ampicillin or kanamycin, incubated for 2 days at 30 °C, and stored frozen at –80 °C in 25% (v/v) glycerol.

Morphological (i.e., Gram reaction, shape, motility, pigments production, respiratory type) (Holt et al. 1994), biochemical (i.e., catalase, oxidase, lactose utilization, API 20 NE assay, bioMérieux, Marcy-l’Étoile, France), and molecular characteristics (i.e., DNA fingerprints, 16S rRNA gene sequencing) of the bacterial isolates were further determined. Genomic DNA was extracted from these bacteria using the Pure Link genomic kit (Invitrogen, Carlsbad, CA, USA).

Polymerase chain reaction (PCR) amplification of 16S rRNA gene was performed in a total reaction volume of 50 μl using GoTaq G2 Flexi DNA polymerase (Promega, Madison, WI, USA) in the supplied buffer. The universal bacterial primers used were 27f and 1492r (Marchesi et al. 1998). Amplifications were carried out in a Mastercycler pro S (Eppendorf, Hamburg, Germany) using the following program: initial denaturation at 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 2 min, with final extension at 72 °C for 10 min. Restriction analyses of the amplified 16S rDNA (ARDRA) were performed with EcoRI, HaeIII, and HhaI (Promega) in a total volume of 20 μl at 37 °C for 3 h. Reaction products and restriction fragments resolved by 2% (w/v) agarose gel electrophoresis (Sambrook and Russel 2001) and stained with SYBR safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) were visualized under ultraviolet (UV) light.

Random amplification of polymorphic DNA (RAPD) fragments was performed in a total reaction volume of 25 μl using GoTaq G2 Flexi DNA polymerase (Promega) in the supplied buffer. The primers used were AP12 (Michaud et al. 2004) and AP5 (Pini et al. 2007). Amplifications were carried out in a Mastercycler pro S (Eppendorf) using the following program: initial denaturation at 94 °C for 10 min, followed by 45 cycles of 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min, with final extension at 72 °C for 10 min. Reaction products were visualized by 2% (w/v) agarose gel electrophoresis (Sambrook and Russel 2001).

Sequencing of the amplified 16S rRNA gene was performed by the CeMIA (SA Larissa, Greece) using the amplification primers (27f and 1492r). The new sequences were aligned and compared to those in GenBank database using the NCBI BLAST algorithm (Altschul et al. 1997).

**Kerosene tolerance of *Achromobacter* and *Pseudomonas* strains**

**Solid medium overlay assay**

The tolerance of isolated bacteria to kerosene was determined by plate overlay assay (Satpute et al. 2008). Overnight cultures, grown in liquid LB medium at 30 °C,
were spotted (20 μl, OD_{660} 0.3) onto LB agar plates, air dried, and then overlaid with kerosene (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Control experiments were set up in the same way, but in the absence of kerosene. Here, as elsewhere in this work, the assays were done in duplicate. The plates with or without kerosene were incubated 1 day at 30 °C. Kerosene tolerance was measured as a function of the bacterial growth (colony formation), as compared with the control. Here, as elsewhere in this work, the Petri plates were visualized under visible light (500 nm) and UV light (366 nm).

**Liquid medium overlay assay**

Overnight cultures grown in liquid LB medium at 30 °C were inoculated (100–200 μl, OD_{660} 0.1) in liquid LB medium, and then overlaid with 5% (v/v) kerosene. Control experiments were set up in the same way, but in the absence of kerosene. Flasks were incubated 1, 3, and 6 days at 30 °C on a rotary shaker (200 rpm). The tolerance of isolated bacteria to kerosene was monitored by determining the optical density at 660 nm (OD_{660}) using a SPECTORD 200 UV-visible spectrophotometer (Analytik Jena, Jena, Germany), the cell viability by the spot method as described by Stancu (2018), and the biomass using the dry weight method (Silva et al. 2010). The degradation of kerosene by isolated bacteria was monitored by gravimetric analyses and by high-performance thin-layer chromatography (HPTLC) analyses of the residual kerosene extracted with chloroform (Gulati and Mehta 2017). HPTLC analyses were carried out using a CAMAG TLC system (Muttenz, Switzerland). The samples were loaded under nitrogen stream on precoated silica gel 60 plates (Merck) and developed using n-hexane-ethyl acetate-methanol-water (40:20:20:4, v/v/v/v) as carrier solution (Eberlin et al. 2009), methanol or n-hexane-ethyl acetate-methanol-water (40:20:20:4, v/v/v/v) as carrier solution. The plates were visualized and scanned under UV light (254 nm) and under visible light (500 nm) after derivatization with anisaldehyde solution (Eberlin et al. 2009).

**Secondary metabolites production by Achromobacter and Pseudomonas strains**

**Surfactants**

Production of extracellular surfactants was monitored by determining the cell growth and the formation of a dark blue halos on CTAB (cetyl trimethyl ammonium bromide) methylene blue agar (Siegmund and Wagner 1991), the emulsification index (E_{24}) for kerosene (Abdel-Mawgoud et al. 2009), and by HPTLC analyses of the crude surfactants extracted with chloroform-methanol (Gesheva et al. 2010). HPTLC analyses were carried out using a CAMAG TLC system (Muttenz). The samples were loaded under nitrogen stream on precoated silica gel 60 plates (Merck) and developed using chloroform-methanol-water (65:25:4, v/v/v) as carrier solution (Gesheva et al. 2010). The plates were visualized and scanned under UV light (254 nm) and under visible light (500 nm) after derivatization with iodine vapors or orcinol solution (Gesheva et al. 2010).

Genomic DNA was extracted using Pure Link genomic kit (Invitrogen). PCR amplification of rhlAB gene was performed in a total reaction volume of 25 μl using GoTag G2 Flexi DNA polymerase (Promega) in the supplied buffer. The primers used were rhlA-f and rhlB-r (Medina et al. 2003). Amplifications were carried out in a Mastercycler pro S (Eppendorf) using the following program: initial denaturation at 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 2 min, with final extension at 72 °C for 10 min. Reaction products resolved by 1.5% (w/v) agarose gel electrophoresis (Sambrook and Russel 2001) and stained with SYBR safe DNA gel stain (Invitrogen) were visualized under UV light.

**Pigments**

Production of extracellular pigments, such as pyocyanin and pyoverdin, was monitored by determining the formation of blue-green and yellow-green fluorescent colonies on King A and King B agar (King et al. 1954) and by HPTLC analyses of the pigments extracted with dichloromethane (Jensen et al. 2006). HPTLC analyses were carried out using a CAMAG TLC system (Muttenz). The samples were loaded under nitrogen stream on precoated silica gel 60 plates (Merck) and developed using dichloromethane-methanol (95:5, v/v) as carrier solution (Jensen et al. 2006). The plates were visualized and scanned under UV light (254 nm). Pyocyanin extracted with chloroform and 0.2 N HCl (Jensen et al. 2006) was quantified by UV-visible (200-800 nm) spectroscopy analyses (El-Fouly et al. 2015) using a SPECTORD 200 UV-visible spectrophotometer (Analytik Jena).

Production of intracellular pigments, such as carotenoid pigments, was monitored by determining the formation of creamy colonies on LB or King B agar and by HPTLC analyses of the pigments extracted with acetone (Beuttler et al. 2011). HPTLC analyses were carried out using a CAMAG TLC system (Muttenz). The samples were loaded under nitrogen stream on precoated silica gel 60 plates (Merck) and developed using chloroform-methanol (90:10, v/v) as carrier solution (Provvedi et al. 2008). The plates were visualized and scanned under UV light (254 nm). Production of carotenoid pigments, such as zeaxanthin, was confirmed by UV-visible (200-800 nm) spectroscopy analyses of crude extracts (Beuttler et al. 2011) using a SPECTORD 200 UV-visible spectrophotometer (Analytik Jena).
Results and discussion
Isolation and characterization of Achromobacter and Pseudomonas strains
As a result of very low growth rate of many bacteria which exist in petroleum and petroleum product-polluted environments, less than 1% of the bacteria are cultivable in laboratory conditions. Thus, only fast growing or the best adapted bacteria are cultivable in laboratory conditions (Chikere et al. 2011). Using a culture-dependent approach, we succeed to isolate five bacterial strains from different samples polluted with petroleum and petroleum products. Strain IBBPo18 was isolated from a water sample, strains IBBPo19 and IBBPo20 were isolated from a soil sample, while strains IBBPo21 and IBBPo22 were isolated from an oily sludge sample.

Distinct morphological and biochemical characteristics were observed for the isolated bacteria (Table 1, Fig. 1). Strains IBBPo18, IBBPo19, and IBBPo21 formed creamy-white pigmented colonies, while strains IBBPo20 and IBBPo22 formed yellow-green pigmented colonies on LB agar. All isolates were Gram-negative, motile, rod-shaped cells, and facultative anaerobic. They were positive for catalase, oxidase, L-arginine dihydrolase, D-glucose, potassium gluconate, capric acid, malic acid, and for trisodium citrate assimilation and negative for lactose, indole, D-glucose fermentation, esculin hydrolysis, β-galactosidase, and for D-maltose assimilation. Furthermore, the isolated bacteria were positive or negative for nitrate reductase, urease, gelatin hydrolysis, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, adipic acid, and for phenylacetic acid assimilation. Based on morphological and biochemical characteristics, all isolates were classified within the genus Achromobacter and Pseudomonas.

The DNA extracted from isolated bacteria was used as template for PCR amplification of 16S rRNA gene using universal bacterial primers, such as 27f and 1492r (Marchesi et al. 1998). The conserved fragment of the 16S rRNA gene with 1465 bp was length in all isolates (Table 1, Fig. 1). ARDRA analyses were carried out on the amplified fragment with EcoRI, HaeIII, and HhaI restriction enzymes. Two ARDRA profiles were obtained when the amplified fragment was digested with EcoRI: one group composed of IBBPo18 and IBBPo21 isolates, and the second group composed of IBBPo19, IBBPo20, and IBBPo22 isolates. When the amplified fragment was digested with HaeIII and HhaI, three ARDRA profiles were obtained: one group composed of IBBPo18 and IBBPo21 isolates, the second group composed of IBBPo19 isolate, and the third group composed of IBBPo20 and IBBPo22 isolates.

For further characterization of the isolated bacteria, DNA extracted from all of them was used as template for RAPD analyses using primers AP12 (Michaud et al. 2004) and AP5 (Pini et al. 2007). As expected, three distinct RAPD profiles were observed: one group composed of IBBPo18 and IBBPo21 isolates, the second group composed of IBBPo19 isolate, and the third group composed of IBBPo20 and IBBPo22 isolates.

The ARDRA and RAPD analyses revealed that the five isolates produced three different DNA fingerprints. Strains IBBPo18, IBBPo19, and IBBPo22 which exhibited different DNA fingerprints were chosen for 16S rRNA gene sequence analyses. The sequences of IBBPo18 (MK934546), IBBPo19 (MK934547), and IBBPo22 (MK934548) isolates were 98-100% similar to the 16S rRNA gene sequences of A. spanius UQ283 (CP034689.1), P. putida YM9 (MK634690.1), and P. aeruginosa PsADMC06 (MK598332.1), respectively. Based on morphological, biochemical, and molecular characteristics, strains IBBPo18 and IBBPo20 were classified within the species A. spanius, strain IBBPo19 was classified within the species P. putida, while strains IBBPo20 and IBBPo22 were classified within the species P. aeruginosa. Two of the isolates (i.e., A. spanius IBBPo18 and IBBPo21) belonged to class Betaproteobacteria, while the other three isolates (i.e., P. putida IBBPo19, P. aeruginosa IBBPo20 and IBBPo22) were from class Gammaproteobacteria. Occurrence of these Proteobacteria in environments polluted with petroleum and petroleum products have been previously reported (Joy et al. 2017).

Kerosene tolerance of Achromobacter and Pseudomonas strains
The tolerance of Achromobacter and Pseudomonas isolates to kerosene was further investigated in both solid and liquid media.

Solid medium plate overlay assay
The ability of the isolated bacteria to tolerate kerosene was first determined by plate overlay assay (Fig. 2a). Lower growth was observed for A. spanius IBBPo18 and IBBPo21 and P. putida IBBPo19 when they were spotted on LB agar overlaid or not with kerosene, compared with that observed for P. aeruginosa IBBPo20 and IBBPo22 which showed profuse growth on the kerosene overlay plates. It was not surprising to observe such differences from one bacterium to another, as the tolerance of bacteria to toxic hydrocarbons is a strain-specific characteristic (Sardessai and Bhosle 2004). If we compare the growth of each Achromobacter and Pseudomonas isolates with their corresponding control, there were no significant differences between bacteria growth on LB agar and their growth on LB overlaid with kerosene. Usually, the tolerance level derived from the growth on nutrient agar media overlaid with hydrocarbons could be different from that observed in two-phase biotransformation systems consisting of a liquid nutrient medium and a hydrocarbon. Several additional parameters, such as
Table 1 Morphological, biochemical, and molecular characteristics of *Achromobacter* and *Pseudomonas* strains

| Characteristics                  | Strain          |
|----------------------------------|-----------------|
|                                  | **IBBPo18**     | **IBBPo19**     | **IBBPo20**     | **IBBPo21**     | **IBBPo22**     |
| Morphological, biochemical       |                 |                 |                 |                 |                 |
| Gram                             | −               | −               | −               | −               | −               |
| Shape                            | Rods            | Rods            | Rods            | Rods            | Rods            |
| Motility                         | +               | +               | +               | +               | +               |
| Color of colonies                | Creamy-white    | Creamy-white    | Yellow-green    | Creamy-white    | Yellow-green    |
| Pyocyanin pigment production     | −               | −               | +               | −               | +               |
| Pyoverdin pigment production     | −               | −               | +               | −               | −               |
| Facultative anaerobic growth     | +               | +               | +               | +               | +               |
| Catalase                         | +               | +               | +               | +               | +               |
| Oxidase                          | +               | +               | +               | +               | +               |
| Lactose utilization              | −               | −               | −               | −               | −               |
| Nitrates reduction               | +               | −               | +               | +               | +               |
| Indole                           | −               | −               | −               | −               | −               |
| D-glucose fermentation           | −               | −               | −               | −               | −               |
| L-arginine dihydrolase           | +               | +               | +               | +               | +               |
| Urease                           | −               | −               | +               | −               | +               |
| Esculin hydrolysis               | −               | −               | −               | −               | −               |
| Gelatin hydrolysis               | −               | −               | +               | −               | −               |
| β-galactosidase                  | −               | −               | −               | −               | −               |
| D-glucose fermentation           | +               | +               | +               | +               | +               |
| L-arabinose assimilation         | +               | +               | −               | +               | −               |
| D-mannose assimilation.          | +               | +               | −               | +               | −               |
| D-mannitol assimilation.         | +               | −               | +               | +               | +               |
| N-acetyl-glucosamine assimilation| −               | −               | +               | −               | +               |
| D-maltose assimilation           | −               | −               | −               | −               | −               |
| Potassium gluconate assimilation | +               | +               | +               | +               | +               |
| Capric acid assimilation         | +               | +               | +               | +               | +               |
| Adipic acid assimilation         | +               | −               | +               | +               | +               |
| Malic acid assimilation          | +               | +               | +               | +               | +               |
| Trisodium citrate assimilation   | +               | +               | +               | +               | +               |
| Phenylacetic acid assimilation   | +               | +               | −               | −               | −               |
| Molecular                        |                 |                 |                 |                 |                 |
| ARDRA using                      |                 |                 |                 |                 |                 |
| 27f/1492r primers (DFS, bp)      | 1465            | 1465            | 1465            | 1465            | 1465            |
| EcoRI restriction enzyme (DFS, bp)| 370, 520, 700   |                 |                 |                 |                 |
| HaeIII, HhaI restriction enzymes (DFS, bp)| 300, 350   | 150, 200, 340, 430 | 150, 200, 350 | 300, 350 | 150, 200, 350 |
| RAPD using                       |                 |                 |                 |                 |                 |
| AP12 primer (DFS, bp)            | 400–2400        | 390–3000        | 360–3000        | 400–2400        | 360–3000        |
| APS primer (DFS, bp)             | 300–1900        | 480–2000        | 270–1450        | 350–2500        | 280–1600        |
| 16S rRNA gene sequence, sequence identity (%) | A. spanius, 99 | P. putida, 98 | P. aeruginosa, ND | A. spanius, ND | P. aeruginosa, 100 |
| GenBank accession number         | MK934546        | MK934547        | −               | −               | MK934548        |

Pyocyanin and pyoverdin (fluorescein) pigment production on King A and King B agar, respectively
DFS detected fragments size, + positive reaction, − negative reaction, ND not determined
Fig. 1 Morphological, biochemical and molecular characteristics of Achromobacter and Pseudomonas strains. a Macroscopic view (MV): A. spanius IBBPo18 (1), P. putida IBBPo19 (2), P. aeruginosa IBBPo20 (3), A. spanius IBBPo21 (4), P. aeruginosa IBBPo22 (5); plates visualized under visible and UV light. b Utilization of several carbon substrates using API 20 NE plates. c Genomic fingerprinting: A. spanius IBBPo18 (1, 6), P. putida IBBPo19 (2, 7), P. aeruginosa IBBPo20 (3, 8), A. spanius IBBPo21 (4, 9), P. aeruginosa IBBPo22 (5, 10); ARDRA PCR of 16S rRNA gene (1465 bp fragment) using 27f/1492r primers (1–5), digestion of the amplified fragment with EcoRI restriction enzyme (6–10); RAPD using AP12 (1–5), AP5 (6–10) primers; 1 kb DNA ladder, Promega (M).

Fig. 2 Kerosene tolerance of Achromobacter and Pseudomonas strains. a Solid medium plate overlay assay (SPOA): A. spanius IBBPo18 (1), P. putida IBBPo19 (2), P. aeruginosa IBBPo20 (3), A. spanius IBBPo21 (4), P. aeruginosa IBBPo22 (5); plates visualized under visible and UV light. b Liquid medium overlay assay (LOA): A. spanius IBBPo18 (1, 2), P. putida IBBPo19 (3, 4), P. aeruginosa IBBPo20 (5, 6), A. spanius IBBPo21 (7, 8), P. aeruginosa IBBPo22 (9, 10), control (1, 3, 5, 7, 9), kerosene (2, 4, 6, 8, 10).
aeration, may possibly be involved in hydrocarbons tolerance in such two-phase systems (Sardessai and Bhosle 2004).

**Liquid medium overlay assay**

We further investigated the ability of the isolated bacteria to tolerate kerosene when they were inoculated in liquid LB medium overlaid with 5% kerosene (Fig. 2b, Fig. 3a–c). All tested bacteria were able to grow in the presence of kerosene. After 1 and 3 days incubation, the growth of *Achromobacter* and *Pseudomonas* isolates in liquid LB medium overlaid or not with kerosene was lower (OD₆₆₀ 0.31–1.27) (data not shown), compared with what acquired after 6 days (OD₆₆₀ 1.39–2.45) (Fig. 3b). We observed that, after 6 days, the growth of *A. spanius* IBBPo18 and IBBPo21 and *P. putida* IBBPo19 in liquid LB medium was lower for the control cells (OD₆₆₀ 1.39–2.03), compared with the growth of the cells in the same medium but overlaid with kerosene (OD₆₆₀ 2.12–2.45). On the contrary, for *P. aeruginosa* IBBPo20 and IBBPo22, the growth was higher for the control cells (OD₆₆₀ 2.27, 2.04), compared with the growth of the cells in the presence of kerosene (OD₆₆₀ 1.71, 1.89). The same trend was observed when the bacterial biomass was determined. The dry weight values for *A. spanius* IBBPo18 and IBBPo21 and *P. putida* IBBPo19 control cells was lower (0.58–1.38 g l⁻¹), compared with those acquired when these bacteria were grown in the presence of kerosene (1.04–1.50 g l⁻¹). Higher dry weight values were obtained for *P. aeruginosa* IBBPo20 and IBBPo22 control cells (1.76, 1.66 g l⁻¹), compared with those obtained when these bacteria were grown in the presence of kerosene (1.71, 1.56 g l⁻¹). Like in the solid medium plate overlay assay, lower growth was observed for *A. spanius* IBBPo18 and IBBPo21 and *P. putida* IBBPo19 when they were spotted on LB agar, compared with what observed for *P. aeruginosa* IBBPo20 and IBBPo22. There were no significant differences between the viability of control cells (confluent cell growth) and the viability of cells grown in the presence of kerosene. The obtained results indicated that the tested bacteria have a good tolerance to kerosene.

Using gravimetric analyses, we further investigated the ability of our bacteria to degrade 5% kerosene (Fig. 3d, e). After 6 days, the degradation of kerosene by *A. spanius* IBBPo18 and IBBPo21 and *P. putida* IBBPo19 was lower (42.0–44.7%), compared with that observed for *P. aeruginosa* IBBPo20 and IBBPo22 (54.2%, 56.9%). It was
not surprising to observe these differences since *P. aeruginosa* strains are the best known bacteria able to utilize a number of aliphatic and aromatic hydrocarbons as the carbon and energy sources (Pacwa-Plociniczak et al. 2014). The ability of other *Achromobacter* (Mazumdar et al. 2015) and *Pseudomonas* (Silva et al. 2006; Ahamed et al. 2010) strains to grow on kerosene as the carbon and energy source was earlier reported. *Achromobacter* sp. AM05 degraded all the major components of kerosene in 60 days (Mazumdar et al. 2015), while *P. alcaligenes* F3.3b degraded 0.2% kerosene in a percent of 76.08% in 7 days (Ahamed et al. 2010). Silva et al. (2006) reported that the maximum growth (2.5 g L⁻¹) for *P. aeruginosa* strain AT18 was in 0.2% kerosene. As observed, our *Achromobacter* and *Pseudomonas* strains degraded 5% kerosene in 6 days with good degradation rates (42.0–56.9%). The degradation of kerosene by the tested bacteria was also confirmed by HPTLC analyses of the residual kerosene extracts. As we described in the “Materials and methods” section, several carrier solution were tested and the best one was n-hexane-ethyl acetate-methanol-water mixture (Fig. 3e). When the TLC plate was visualized under UV light, the control (uninoculated medium overlaid with kerosene) gave two spots at the *Rf* (retardation factor) value of 0.06 and 0.80. These two spots were also detected in extracts from the tested bacteria grown in the presence of kerosene. When the TLC plate was derivatized with anisaldehyde solution and visualized under visible light (data not shown), some of the spots at the *Rf* values of 0.06 and 0.80 were detected in extracts from *Achromobacter* sp. AM05. These two spots were also detected in extracts from *Achromobacter* sp. AM05. These two spots were also detected in extracts from the tested bacteria grown in the presence of kerosene. After 6 days, lower growth on CTAB methylene blue agar was observed for *A. spanius* IBBP018 and IBBP021 and *P. putida* IBBP019, compared with *P. aeruginosa* IBBP020 and IBBP022. Although all tested bacteria grew on CTAB methylene blue agar, the formation of a dark blue halo around the colony (which indicates the production of the extracellular glycolipids or other anionic surfactants) was observed only for *P. aeruginosa* IBBP020 and IBBP022. An increase in biosurfactant production was observed for *P. aeruginosa* IBBP020 and IBBP022 after 6 days incubation, compared with that observed after 1 and 3 days. Our results are in agreement with that earlier reported concerning the overproduction of glycolipid surfactants by *P. aeruginosa* strains during nutrient limitation conditions (Chrzanoski et al. 2012).

As could be observed (Fig. 4b), *Achromobacter* and *Pseudomonas* isolates were able to produce surfactants that emulsify the kerosene, and these tension-active compounds have very good emulsification activities (*E*₂₄ of 57–94%). Mnif et al. (2011) and Joy et al. (2017) reported the isolation of other *Achromobacter* (i.e., *A. xylosoxidans* C350R, *Achromobacter* sp. PS1) and *Pseudomonas* (i.e., *P. aeruginosa* C450R, *Pseudomonas* sp. MRBSIT1) strains with very good emulsification activities (*E*₂₄ of 45–77%). We observed an increase in the emulsification activity (*E*₂₄ of 69–94%) when *Achromobacter* and *Pseudomonas* isolates were grown in the presence of kerosene, as compared with their corresponding controls (*E*₂₄ of 57–86%). The increase in the emulsification activity could be due to the biosurfactant production which has the ability to emulsify the hydrophobic compounds, such as hydrocarbons, and make them more accessible for bacterial degradation (Mnif et al. 2011).

The production of extracellular glycolipid surfactants by the isolated bacteria was confirmed by HPTLC analyses of the crude extracts. When the TLC plate was visualized under UV light (Fig. 4c), several surfactant fractions were observed for *Achromobacter* and *Pseudomonas* isolates, and the number of these fractions varies from one bacterium to another, and even for the same bacterium depending on the culture conditions (growth in liquid medium overlaid or not with kerosene). Between two and four fractions with *Rf* values of 0.41–0.62 were detected in extracts from *A. spanius* IBBP018 and IBBP021, *P. putida* IBBP019, and *P. aeruginosa* IBBP020 and IBBP022. From all these fractions, only two of them, those with *Rf* values of 0.46–0.48 and 0.51–0.53, were detected in all bacteria. When the same TLC plate was derivatized with iodine vapors or orcinol solution and visualized under visible light (data not shown), some of
these spots showed positive reaction indicating the presence of lipids and glycolipids in the surfactant molecules. The HPTLC analyses confirmed that surfactants produced by *Achromobacter* and *Pseudomonas* isolates are glycolipids; however, on the CTAB methylene blue agar assay, the formation of a dark blue halo around the colony was observed only for *P. aeruginosa* isolates. Like other *Pseudomonas* strains (e.g., *P. aeruginosa*, *P. putida*) (Rikalović et al. 2015), our *Pseudomonas* isolates (*P. putida* IBBPo19, *P. aeruginosa* IBBPo20 and IBBPo22) produced glycolipid surfactants, which are probably rhamnolipids in the case of *P. aeruginosa* isolates. Furthermore, surfactants produced by *Achromobacter* isolates (*A. spanius* IBBPo18 and IBBPo21) are also glycolipids. Similarly, Joy et al. (2017) reported glycolipid surfactant production by *Achromobacter* sp. PS1.

Another assay for the determination of the ability of the isolated bacteria to produce surfactants is the detection of enzyme-encoding genes involved in their biosynthesis (Pacwa-Plociniczak et al. 2014). Since all *Achromobacter* and *Pseudomonas* isolates were able to produce glycolipid surfactants, we further checked the presence of *rhlAB* (rhamnosyl transferase) gene in their genome. DNA extracted from bacteria grown 1 and 6 days in liquid LB medium overlaid or not with 5% kerosene was used as template for PCR amplification of *rhlAB* gene (Fig 5a, b) using primers rhlA-f and rhlB-r (Medina et al. 2003). As expected, the fragment of the *rhlAB* gene with 216 bp in length was detected in higher quantities only in the DNA extracted from *P. aeruginosa* IBBPo20 and IBBPo22; no significant changes in their PCR pattern were observed. On the contrary, in the DNA extracted from *A. spanius* IBBPo18 and IBBPo21, the *rhlAB* gene was detected in barely quantities and changes in their PCR pattern were observed. In the DNA extracted from *P. putida* IBBPo19, the detected fragment has higher size (approximately 220–230 bp in length), as compared with the fragment of the *rhlAB* gene detected in *P. aeruginosa* IBBPo20 and IBBPo22 (216 bp); no significant changes in the PCR pattern were observed in the DNA extracted from *P. putida* IBBPo19. The PCR of *rhlAB* gene confirmed the production of anionic rhamnolipid surfactants by *P. aeruginosa* IBBPo20 and IBBPo22. Due to their characteristics (e.g., high affinity for hydrophobic molecules, high emulsifying...
activity, low critical micelle concentration values), surfactants and especially glycolipid biosurfactants, such as rhamnolipids, were reported to improve the biodegradation of many petroleum and petroleum products (Rocha et al. 2011; Chrzanowski et al. 2012).

**Pigments**

The ability of the isolated bacteria to produce extracellular pigments, such as pyocyanin and pyoverdin pigments, was confirmed by determining the formation of blue-green colonies on King A and yellow-green fluorescent colonies on King B. The ability of the isolated bacteria to produce extracellular pigments, such as pyocyanin and pyoverdin pigments, was confirmed by determining the formation of blue-green colonies on King A and yellow-green fluorescent colonies.

![Fig. 5 Detection of rhlAB gene in DNA extracted from Achromobacter and Pseudomonas strains. a PCR using DNA extracted after 1 day incubation. b PCR using DNA extracted after 6 days incubation. A. spanius IBBPo18 (1, 2), P. putida IBBPo21 (3, 4), P. aeruginosa IBBPo22 (5, 6), A. spanius IBBPo20 (7, 8), P. aeruginosa IBBPo20 (9, 10), control (1, 3, 5, 7, 9), kerosene (2, 4, 6, 8, 10); PCR of rhlAB gene (216 bp fragment) using rhlA-f/rhlB-r primers (1–10); 1 kb DNA ladder, Promega (M).](image)

![Fig. 6 Extracellular pigments production by Achromobacter and Pseudomonas strains. a Pyocyanin (Pc) production: A. spanius IBBPo18 (1, 2), P. putida IBBPo19 (3, 4), P. aeruginosa IBBPo20 (5, 6), A. spanius IBBPo21 (7, 8), P. aeruginosa IBBPo22 (9, 10), control (1, 3, 5, 7, 9), kerosene (2, 4, 6, 8, 10); plates visualized under visible and UV light. b Pyocyanin quantification (PcQ) assay: concentration (µg ml⁻¹) = OD₅₂₀ × 17.072, not detected (ND). The values represent the average from two independent assays (µg ml⁻¹) with standard deviation (SD). c HPTLC: A. spanius IBBPo18 (1, 2), P. putida IBBPo19 (3, 4), P. aeruginosa IBBPo20 (5, 6), A. spanius IBBPo21 (7, 8), P. aeruginosa IBBPo22 (9, 10), control (1, 3, 5, 7, 9), kerosene (2, 4, 6, 8, 10); pigments standards (Sp), 2-heptyl-4-hydroxy-quinolone (HHQ), Pseudomonas quinolone signal (PQS); plate visualized and scanned under UV light.](image)
on King B agar, respectively. Although all tested bacteria were able to grow on these agar media, the pyocyanin (Fig. 6a) and/or pyoverdin pigments production was observed only for *P. aeruginosa* isolates. *P. aeruginosa* IBBPo20 produced both pyocyanin and pyoverdin pigments, while *P. aeruginosa* IBBPo22 produced only pyocyanin. The ability of these two *P. aeruginosa* strains to produce pigments varies from one bacterium to another, and even for the same bacterium depending on the culture conditions. The pyocyanin pigments extracted from *P. aeruginosa* IBBPo20 and IBBPo22 showed characteristic peaks at 205–210, 275, 390, and 525 nm, and the pigment production significantly decreased from 32.40 to 0.30 μg ml⁻¹ and from 74.96 to 3.89 μg ml⁻¹, respectively, when these bacteria were grown in the presence of kerosene (Fig. 6b). Furthermore, significant differences between UV-Vis absorption spectra of the pyocyanin pigments extracted from the bacterial cells grown in the presence of kerosene were observed, as compared with their corresponding controls (data not shown). In the acidic form, pyocyanin pigments extracted from other *Pseudomonas* strains (i.e., *P. aeruginosa* R₆, *P. aeruginosa* U₃) showed characteristic peaks at 300, 388, and 518 nm (El-Fouly et al. 2015). Hydroxy-alkyl-quinoline (HAQ) metabolite production by tested bacteria was monitored by HPTLC analyses of the crude pigment extracts. When the TLC plate was visualized under UV light (Fig. 6c), several fractions with Rₐ values of 0.04–0.50 were observed in extracts from *A. spanius* IBBPo18 and IBBPo21, *P. putida* IBBPo19, and *P. aeruginosa* IBBPo20 and IBBPo22, and the number of these fractions varies from one bacterium to another. The spot with Rₐ values of 0.04–0.06 and 0.18–0.21 correspond probably to HAQs metabolites, such as 2-heptyl-4-hydroxy-quinolone (HHQ) and 2-heptyl-3-hydroxy-4-quinolone or *Pseudomonas* quinolone signal (PQS), respectively. All our bacteria were able to produce HHQ, while the production of PQS was observed only in *P. aeruginosa* IBBPo20 and IBBPo22. According with literature, PQS and its precursor HHQ are secondary metabolites produced by various bacteria, including by *Pseudomonas* sp. strains which act as signaling molecules in cell-to-cell communication in the quorum
sensing system (Jensen et al. 2006; Niewerth et al. 2011). PQS signaling is directly or indirectly involved in regulation of secondary metabolite production (e.g., pigments, surfactants) and virulence factor production, biofilm formation, motility, and also in membrane vesicle formation. HHQ and PQS exhibit also a very good antimicrobial activity against various Gram-positive and Gram-negative bacteria (Jensen et al. 2006; Niewerth et al. 2011; Reen et al. 2011). The isolation of new bacteria able to produce HAQs metabolites is very important and will make possible the discovery of new therapeutic strategies to combat microorganisms which are refractory to conventional antimicrobial agents (Reen et al. 2011).

The ability of the isolated bacteria to produce intracellular pigments, such as carotenoids, was also investigated. Creamy pigments which showed absorption maxima between 340 and 355 nm were produced by A. spanius IBBPo18 and IBBPo21, P. putida IBBPo19, and P. aeruginosa IBBPo20 and IBBPo22 (Fig. 7a, b). According with literature (Sajilata et al. 2008), cis-zeaxanthin isomers show absorption in the near-UV region (320–340 nm), while trans-isomers absorb in the visible region (400–500 nm). Significant differences between UV-Vis absorption spectra of the carotenoid pigments extracted from the bacterial cells grown in the presence of kerosene were observed, as compared with their corresponding controls (data not shown). Production of carotenoids by Achromobacter and Pseudomonas isolates was monitored also by HPTLC analyses of the crude pigment extracts. When the TLC plate was visualized under UV light (Fig. 7c), several fractions with \( R_f \) values of 0.06–0.58 were observed in extracts from A. spanius IBBPo18 and IBBPo21, P. putida IBBPo19, and P. aeruginosa IBBPo20 and IBBPo22, and the number of these fractions varies from one bacterium to another. The spots with \( R_f \) values of 0.10–0.16 which correspond probably to zeaxanthin (Zx or \( \beta \beta \)-carotene-3,3′-diol) were detected in all the isolated bacteria extracts, while the spots with \( R_f \) values of 0.57–0.58 which correspond to \( \beta \)-carotene (BC) were detected only in P. aeruginosa extracts.

Pigments produced by different bacteria have been extensively used in food and textile industries, paper production, agricultural practices, water science, and technology (Malik et al. 2012; Usman et al. 2017). Furthermore, the pigments (e.g., pyocyanin, carotenoids) which showed useful biological activities, such as antioxidants, antibiotic, and anticancer agents, have also been used in medicine (Malik et al. 2012; El-Fouly et al. 2015; Usman et al. 2017).

**Conclusions**

Five Proteobacteria from Beta- and Gamma- classes were isolated from different samples polluted with petroleum and petroleum products. Based on their morphological, biochemical, and molecular characteristics, isolated bacteria were identified as A. spanius strains IBBPo18 and IBBPo21, P. putida strain IBBPo19, and P. aeruginosa strains IBBPo20 and IBBPo22. All the isolated bacteria were able to tolerate and degrade kerosene, and their tolerance and degradation rates vary from one bacterium to another. Achromobacter and Pseudomonas isolates were able to produce several secondary metabolites (i.e., surfactants, pigments), and their production varies from one bacterium to another. Glycolipid surfactants produced by Achromobacter and Pseudomonas isolates have a very good emulsification activity, and their activity increased in the presence of kerosene. Pyocyanin and pyoverdin pigments were produced only by P. aeruginosa IBBPo20 and IBBPo22 while carotenoid pigments were produced by all the isolated bacteria. Significant changes in pigment production were observed when Achromobacter and Pseudomonas isolates were grown in the presence of kerosene. Due to their ability to tolerate and degrade kerosene, and also to produce secondary metabolites, the bacteria which were isolated in this study could be used in the bioremediation of environments polluted with complex mixtures of hydrocarbons, such as kerosene.

**Acknowledgements**

The author is grateful to Ana Dinu for technical support.

**Authors’ contributions**

The author read and approved the final manuscript.

**Funding**

The study was funded by project no. RO1567-IBB05/2019 from the Institute of Biology Bucharest of Romanian Academy.

**Ethics approval and consent to participate**

This article does not contain any studies with human participants or laboratory animals.

**Consent for publication**

Not applicable.

**Competing interests**

The author declares that there are no competing interests.

**Received**: 30 September 2019 **Accepted**: 8 January 2020

**References**

Abdel-Mawgoud AM, Aboulwafa MM, Hassouna NAH (2009) Characterization of rhamnolipid produced by Pseudomonas aeruginosa isolate Bs20. Appl Biochem Biotechnol 157:329–345

Ahamed F, Hasibullah M, Ferdouse J, Anwar MN (2010) Microbial degradation of petroleum hydrocarbon. Bangladesh J Microbiol 27:10–13

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402

Beutler H, Hoffmann J, Jeske M, Hauer B, Schmid RD, Altenbuchner J, Urlacher VB (2011) Biosynthesis of zeaxanthin in recombinant Pseudomonas putida. Appl Microbiol Biotechnol 89:1137–1147

Chikere CB, Olpokwasili GC, Chikere BO (2011) Monitoring of microbial hydrocarbon remediation in the soil. 3. Biotech 1:117–138

Chrzanowski Ł, Ławniczak Ł, Czaczyk K (2012) Why do microorganisms produce rhamnolipids? World J Microbiol Biotechnol 28:401–419

Ahamed F, Hasibullah M, Ferdouse J, Anwar MN (2010) Microbial degradation of petroleum hydrocarbon. Bangladesh J Microbiol 27:10–13

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402

Beutler H, Hoffmann J, Jeske M, Hauer B, Schmid RD, Altenbuchner J, Urlacher VB (2011) Biosynthesis of zeaxanthin in recombinant Pseudomonas putida. Appl Microbiol Biotechnol 89:1137–1147

Chikere CB, Olpokwasili GC, Chikere BO (2011) Monitoring of microbial hydrocarbon remediation in the soil. 3. Biotech 1:117–138

Chrzanowski Ł, Ławniczak Ł, Czaczyk K (2012) Why do microorganisms produce rhamnolipids? World J Microbiol Biotechnol 28:401–419
