



# Kerosene tolerance in *Achromobacter* and *Pseudomonas* species

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## 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* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>, *Pseudomonas putida* IBB<sub>Po19</sub>, and *Pseudomonas aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>.

**Results:** All these bacteria were able to tolerate and degrade kerosene. Higher tolerance to kerosene and degradation rates were observed for *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>, compared with that observed for *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>, and *P. putida* IBB<sub>Po19</sub>. All these bacteria were able to produce several secondary metabolites, such as surfactants and pigments. Glycolipid surfactants produced by *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>, *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>, and *P. putida* IBB<sub>Po19</sub> 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* IBB<sub>Po20</sub> and IBB<sub>Po22</sub> was confirmed by detection of *rhlAB* gene involved in their biosynthesis. Pyocyanin and pyoverdine pigments were produced only by *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>, while carotenoid pigments were produced by all the isolated bacteria. Significant changes in pigments production were observed when *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>, *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>, and *P. putida* IBB<sub>Po19</sub> 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

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degrade different hydrocarbons have been reported (Leahy and Colwell 1990; Chikere et al. 2011; Mnif et al. 2011; Varjani 2017; Xu et al. 2018; Shahzadi et al. 2019). Most of the bacteria able to tolerate and degrade toxic hydrocarbons were isolated mainly from the 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 degrading ability 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 ( $C_6$ – $C_{17}$ ), including toxic compounds, such as benzene, toluene, trimethylbenzene, ethylbenzene, xylenes, *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 any 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  $\mu\text{g ml}^{-1}$  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'Etoile, 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  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu$ l, OD<sub>660</sub> 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  $\mu$ l, OD<sub>660</sub> 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<sub>660</sub>) using a SPECORD 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 (Muttenez, Switzerland). The samples were loaded under nitrogen stream on precoated silica gel 60 plates (Merck, Darmstadt, Germany) and developed using *n*-hexane-ethyl acetate (92:8, v/v) (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 (Siegmond 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 (Muttenez). 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  $\mu$ l using GoTaq 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 pyoverdine, 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 (Muttenez). 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 SPECORD 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 (Muttenez). 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 (Proveddi 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 SPECORD 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 succeeded to isolate five bacterial strains from different samples polluted with petroleum and petroleum products. Strain IBB<sub>Po18</sub> was isolated from a water sample, strains IBB<sub>Po19</sub> and IBB<sub>Po20</sub> were isolated from a soil sample, while strains IBB<sub>Po21</sub> and IBB<sub>Po22</sub> were isolated from a oily sludge sample.

Distinct morphological and biochemical characteristics were observed for the isolated bacteria (Table 1, Fig. 1). Strains IBB<sub>Po18</sub>, IBB<sub>Po19</sub>, and IBB<sub>Po21</sub> formed creamy-white pigmented colonies, while strains IBB<sub>Po20</sub> and IBB<sub>Po22</sub> 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,  $\beta$ -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 in length was detected 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 IBB<sub>Po18</sub> and IBB<sub>Po21</sub> isolates, and the second group composed of IBB<sub>Po19</sub>, IBB<sub>Po20</sub>, and IBB<sub>Po22</sub> isolates. When the amplified fragment was digested with *HaeIII* and *HhaI*, three ARDRA profiles were obtained: one group composed of IBB<sub>Po18</sub> and IBB<sub>Po21</sub> isolates, the second group composed of IBB<sub>Po19</sub> isolate, and the third group composed of IBB<sub>Po20</sub> and IBB<sub>Po22</sub> 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 IBB<sub>Po18</sub> and IBB<sub>Po21</sub> isolates, the second group composed of IBB<sub>Po19</sub> isolate, and the third group composed of IBB<sub>Po20</sub> and IBB<sub>Po22</sub> isolates.

The ARDRA and RAPD analyses revealed that the five isolates produced three different DNA fingerprints. Strains IBB<sub>Po18</sub>, IBB<sub>Po19</sub>, and IBB<sub>Po22</sub> which exhibited different DNA fingerprints were chosen for 16S rRNA gene sequence analyses. The sequences of IBB<sub>Po18</sub> (MK934546), IBB<sub>Po19</sub> (MK934547), and IBB<sub>Po22</sub> (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 IBB<sub>Po18</sub> and IBB<sub>Po21</sub> were classified within the species *A. spanius*, strain IBB<sub>Po19</sub> was classified within the species *P. putida*, while strains IBB<sub>Po20</sub> and IBB<sub>Po22</sub> were classified within the species *P. aeruginosa*. Two of the isolates (i.e., *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>) belonged to class *Betaproteobacteria*, while the other three isolates (i.e., *P. putida* IBB<sub>Po19</sub>, *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>) 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.

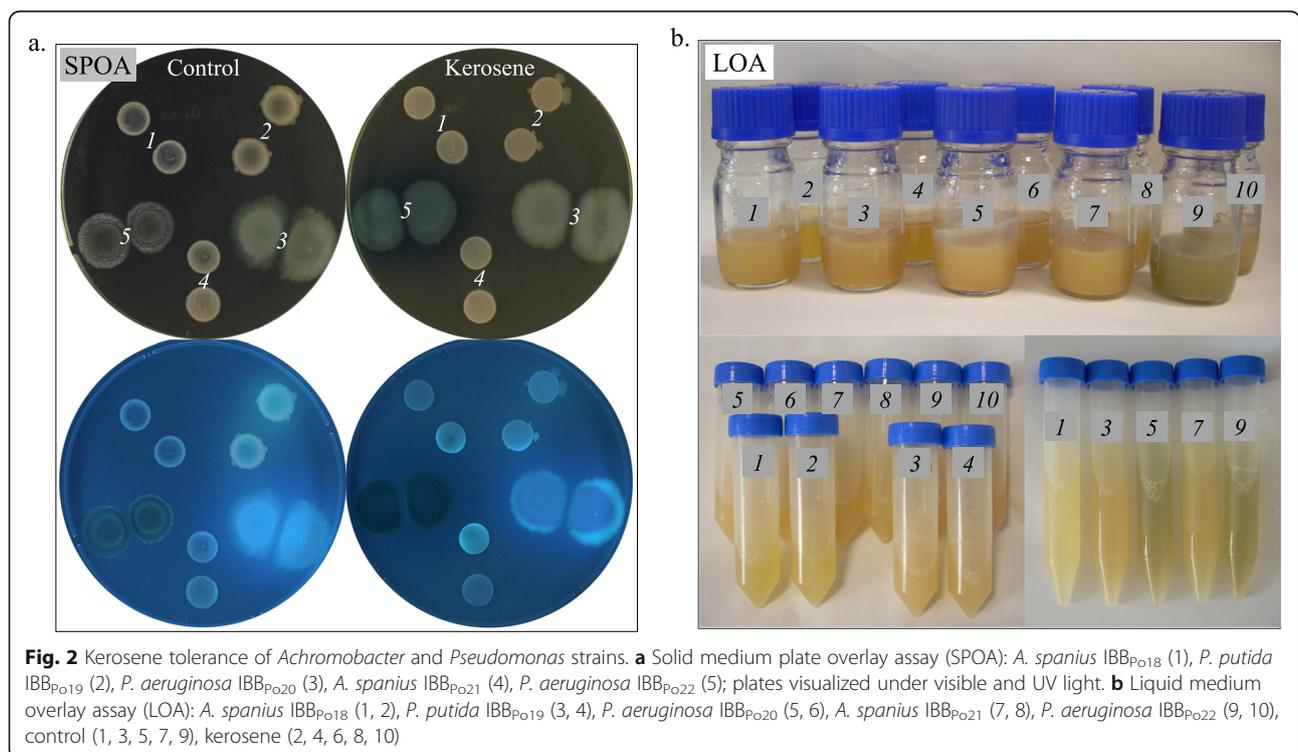
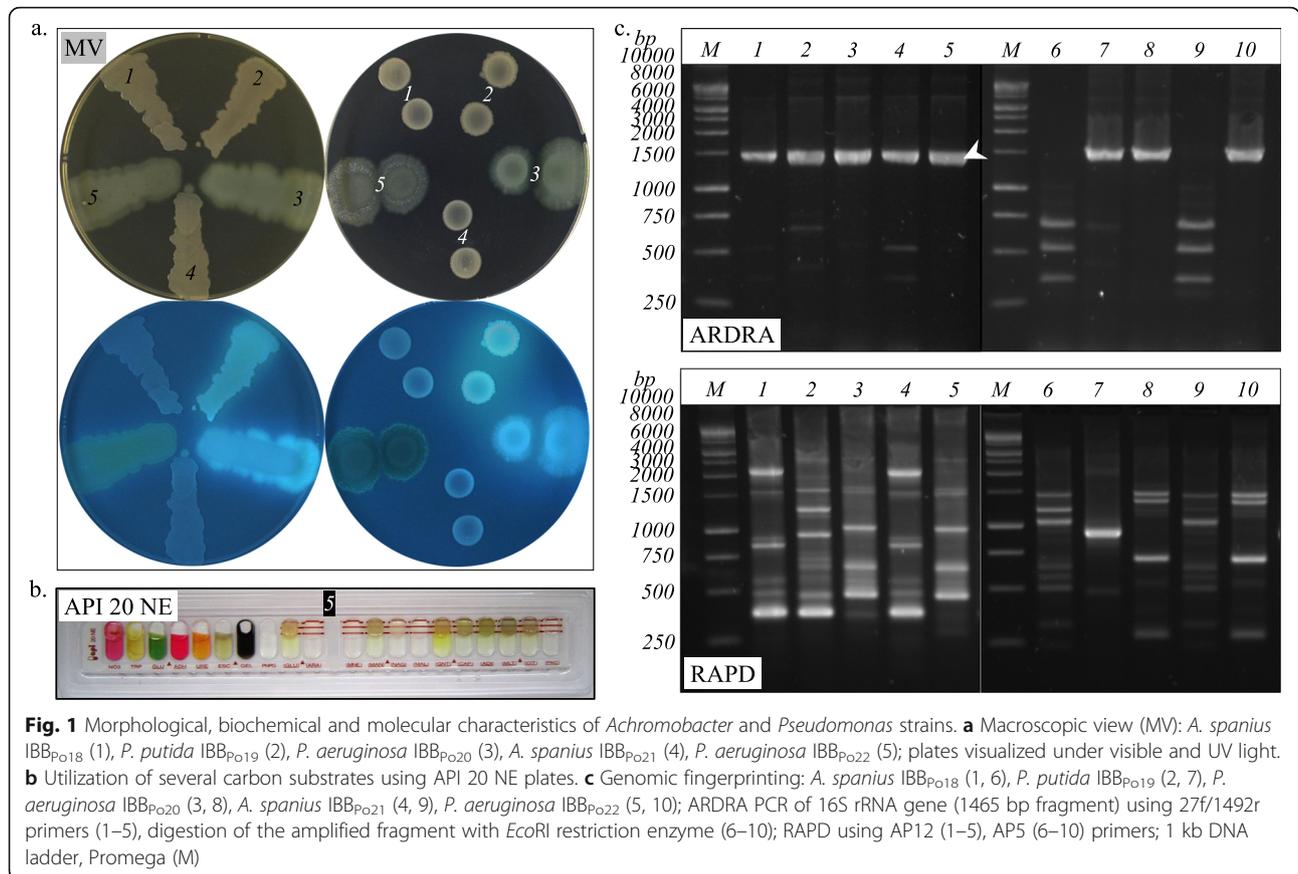
#### Solid medium plate overlay assay

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* IBB<sub>Po18</sub> and IBB<sub>Po21</sub> and *P. putida* IBB<sub>Po19</sub> when they were spotted on LB agar overlaid or not with kerosene, compared with that observed for *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub> 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				
		IBB <sub>Po18</sub>	IBB <sub>Po19</sub>	IBB <sub>Po20</sub>	IBB <sub>Po21</sub>	IBB <sub>Po22</sub>
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 assimilation	+	+	+	+	+
	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
	<i>Eco</i> RI restriction enzyme (DFS, bp)	370, 520, 700	–	–	370, 520, 700	–
	<i>Hae</i> III, <i>Hha</i> I 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
	AP5 primer (DFS, bp)	300–1900	480–2000	270–1450	350–2500	280–1600
	16S rRNA gene sequence, sequence identity (%)	<i>A. spanius</i> , 99	<i>P. putida</i> , 98	<i>P. aeruginosa</i> , ND	<i>A. spanius</i> , ND	<i>P. aeruginosa</i> , 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



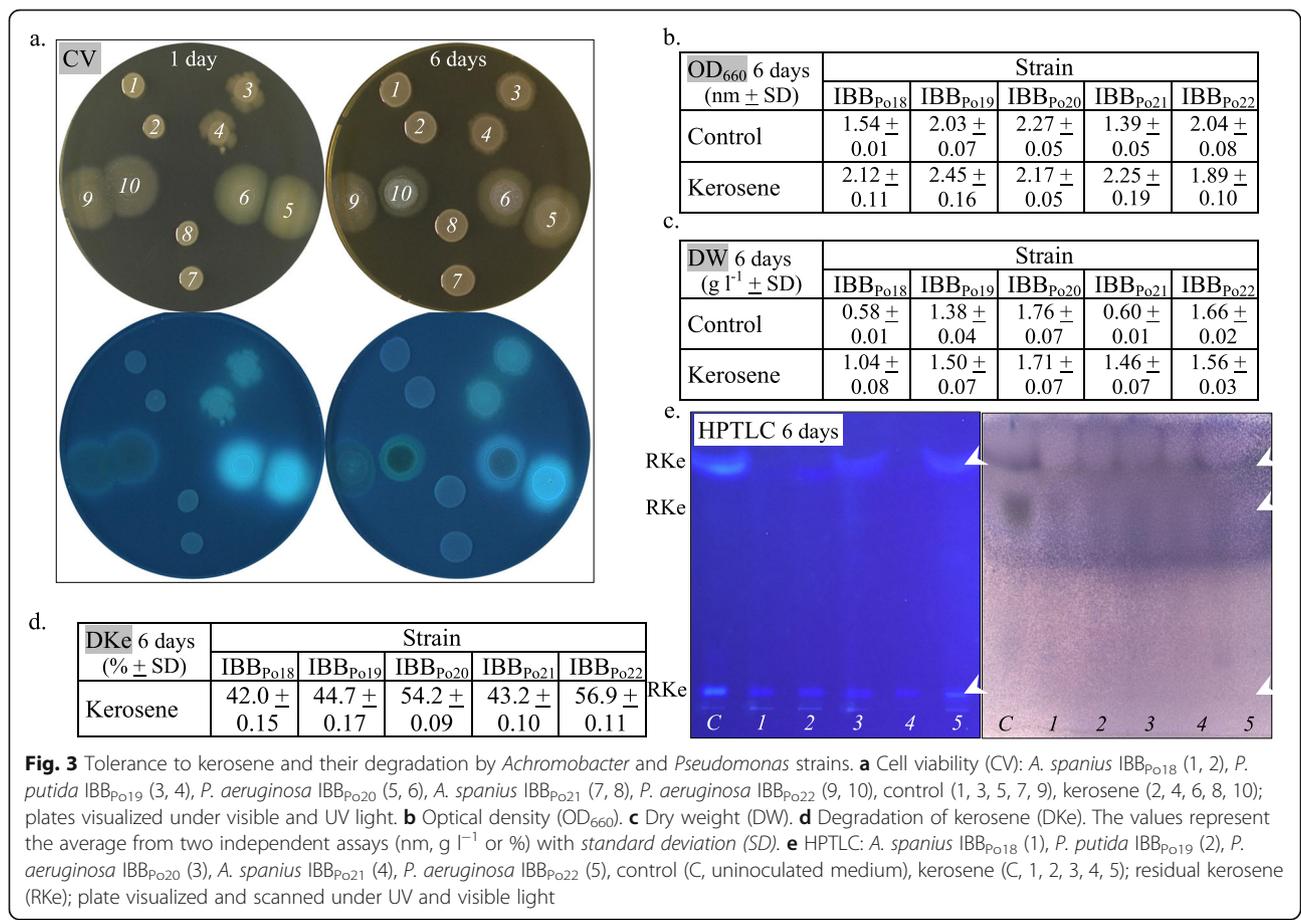
aeration, may possibly be involved in hydrocarbons tolerance in such two-phase systems (Sardessai and Bhosle 2004).

**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 (OD<sub>660</sub> 0.31–1.27) (data not shown), compared with that acquired after 6 days (OD<sub>660</sub> 1.39–2.45) (Fig. 3b). We observed that, after 6 days, the growth of *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub> and *P. putida* IBB<sub>Po19</sub> in liquid LB medium was lower for the control cells (OD<sub>660</sub> 1.39–2.03), compared with the growth of the cells in the same medium but overlaid with kerosene (OD<sub>660</sub> 2.12–2.45). On the contrary, for *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>, the growth was higher for the control cells (OD<sub>660</sub> 2.27, 2.04), compared with the growth of the cells in the presence of kerosene (OD<sub>660</sub> 2.17, 1.89). The same trend was observed when the bacterial biomass

was determined. The dry weight values for *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub> and *P. putida* IBB<sub>Po19</sub> control cells was lower (0.58–1.38 g l<sup>-1</sup>), compared with those acquired when these bacteria were grown in the presence of kerosene (1.04–1.50 g l<sup>-1</sup>). Higher dry weight values were obtained for *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub> control cells (1.76, 1.66 g l<sup>-1</sup>), compared with those obtained when these bacteria were grown in the presence of kerosene (1.71, 1.56 g l<sup>-1</sup>). Like in the solid medium plate overlay assay, lower growth was observed for *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub> and *P. putida* IBB<sub>Po19</sub> when they were spotted on LB agar, compared with that observed for *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>. 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* IBB<sub>Po18</sub> and IBB<sub>Po21</sub> and *P. putida* IBB<sub>Po19</sub> was lower (42.0–44.7%), compared with that observed for *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub> (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-Płociniczak 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. AM<sub>05</sub> degraded all the major components of kerosene in 60 days (Mazumdar et al. 2015), while *P. alcaligenes* F.S3b 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<sup>-1</sup>) 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 *R<sub>f</sub>* (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, an additional spot with the *R<sub>f</sub>* value of 0.66 was detected in the control, while in the extracts from the tested bacteria, this spot was detected only in barely quantities. The presence of these three spots in lower quantities in extracts from the inoculated medium overlaid with kerosene indicates the degradation of some hydrocarbons which exist in the composition of kerosene by tested bacteria.

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

The ability of the isolated bacteria to produce secondary metabolites such as surfactants and pigments when they were inoculated in liquid LB medium overlaid or not with 5% kerosene was also investigated in this study.

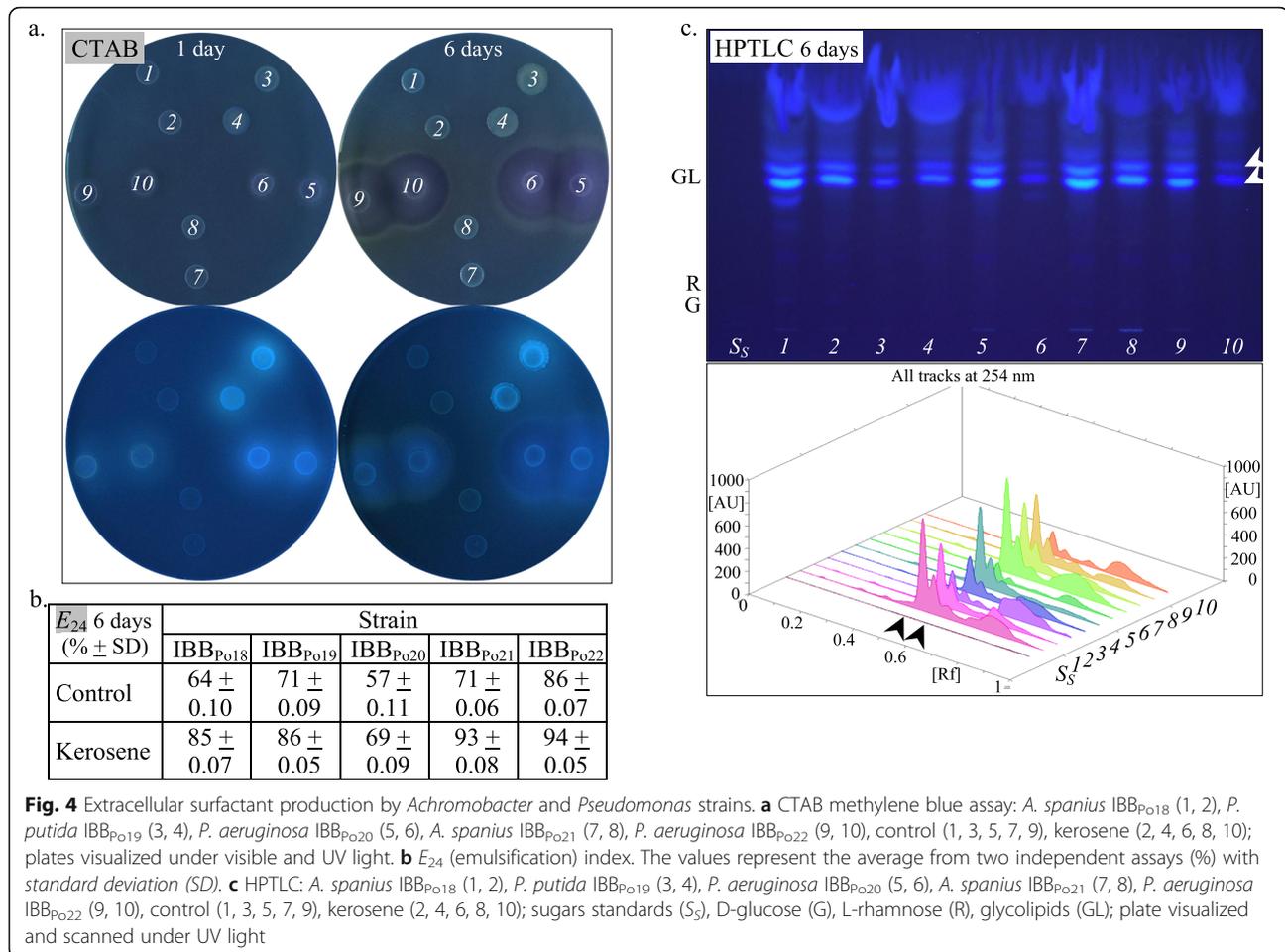
#### Surfactants

One of the numerous physiological adaptations of bacteria induced by presence of hydrocarbons is surfactant production (Joy et al. 2017), which could be present on the cell surface and/or excreted extracellularly (Rikalović et al. 2015). The ability of the isolated bacteria to produce extracellular surfactants was confirmed by CTAB methylene blue agar assay (Fig. 4a). All tested bacteria were able to grow on CTAB methylene blue agar. No

significant differences were observed between the growth of control cells and that observed for the cells grown in the presence of kerosene. After 6 days, lower growth on CTAB methylene blue agar was observed for *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub> and *P. putida* IBB<sub>Po19</sub>, compared with *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>. 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* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>. An increase in biosurfactant production was observed for *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub> 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 (Chrzanowski 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<sub>24</sub>* 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<sub>24</sub>* of 45–77%). We observed an increase in the emulsification activity (*E<sub>24</sub>* of 69–94%) when *Achromobacter* and *Pseudomonas* isolates were grown in the presence of kerosene, as compared with their corresponding controls (*E<sub>24</sub>* 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).

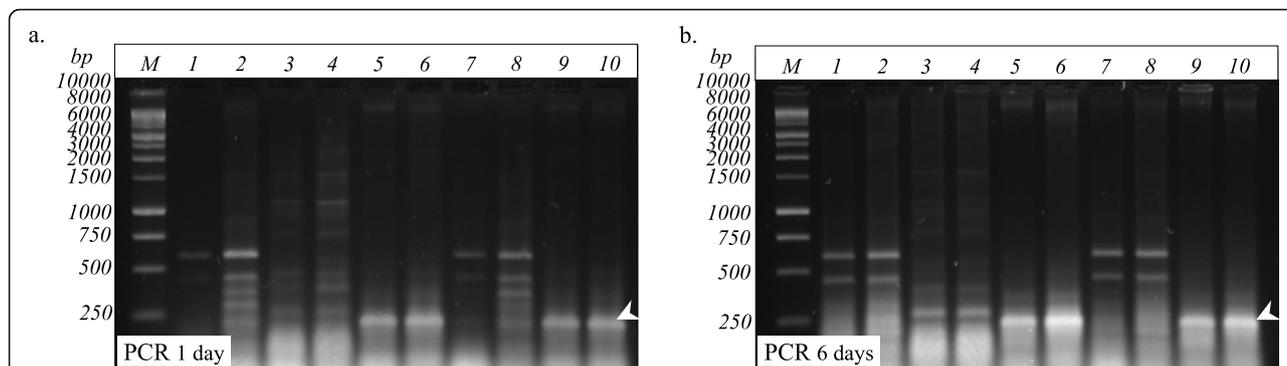
The production of extracellular glycolipid surfactants by the isolated bacteria was confirmed by HPTLC analyses of the crude extracts. When the TLC plate was visualized under UV light (Fig. 4c), several surfactant fractions were observed for *Achromobacter* and *Pseudomonas* isolates, and the number 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 *R<sub>f</sub>* values of 0.41–0.62 were detected in extracts from *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>, *P. putida* IBB<sub>Po19</sub>, and *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>. From all these fractions, only two of them, those with *R<sub>f</sub>* 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* IBB<sub>Po19</sub>, *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>) produced glycolipid surfactants, which are probably rhamnolipids in the case of *P. aeruginosa* isolates. Furthermore, surfactants produced by *Achromobacter* isolates (*A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>) 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-Płociniczak et al. 2014). Since all *Achromobacter* and *Pseudomonas* isolates were able to produce glycolipid surfactants, we further checked the presence of *rhlAB*

(rhamnolipid synthase) 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* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>; no significant changes in their PCR pattern were observed. On the contrary, in the DNA extracted from *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>, the *rhlAB* gene was detected in barely quantities and changes in their PCR pattern were observed. In the DNA extracted from *P. putida* IBB<sub>Po19</sub>, 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* IBB<sub>Po20</sub> and IBB<sub>Po22</sub> (216 bp); no significant changes in the PCR pattern were observed in the DNA extracted from *P. putida* IBB<sub>Po19</sub>. The PCR of *rhlAB* gene confirmed the production of anionic rhamnolipid surfactants by *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>. Due to their characteristics (e.g., high affinity for hydrophobic molecules, high emulsifying

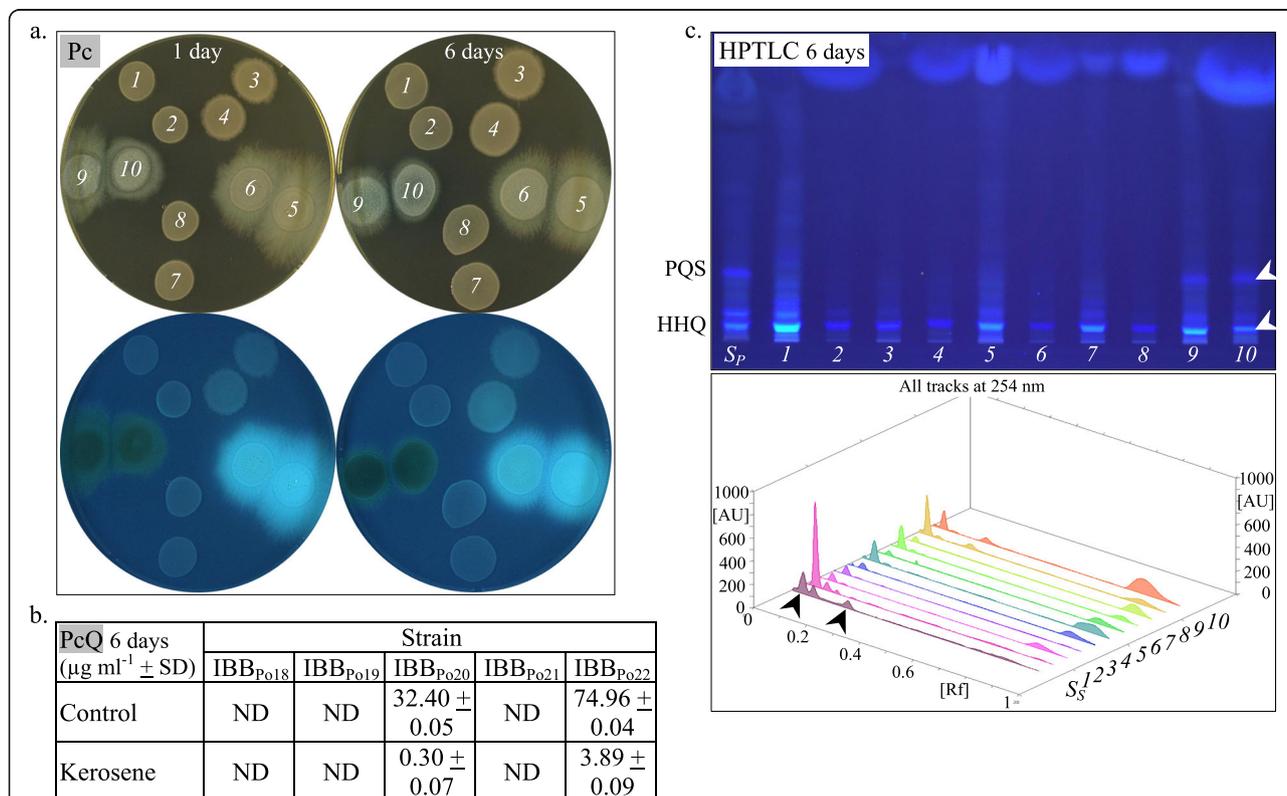


**Fig. 5** Detection of *rhIAB* gene in DNA extracted from *Achromobacter* and *Pseudomonas* strains. **a** PCR using DNA extracted after 1 day incubation. **b** PCR using DNA extracted after 6 days incubation. *A. spanius* IBB<sub>Po18</sub> (1, 2), *P. putida* IBB<sub>Po19</sub> (3, 4), *P. aeruginosa* IBB<sub>Po20</sub> (5, 6), *A. spanius* IBB<sub>Po21</sub> (7, 8), *P. aeruginosa* IBB<sub>Po22</sub> (9, 10), control (1, 3, 5, 7, 9), kerosene (2, 4, 6, 8, 10); PCR of *rhIAB* gene (216 bp fragment) using *rhIA-f/rhIB-r* primers (1–10); 1 kb DNA ladder, Promega (M)

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**

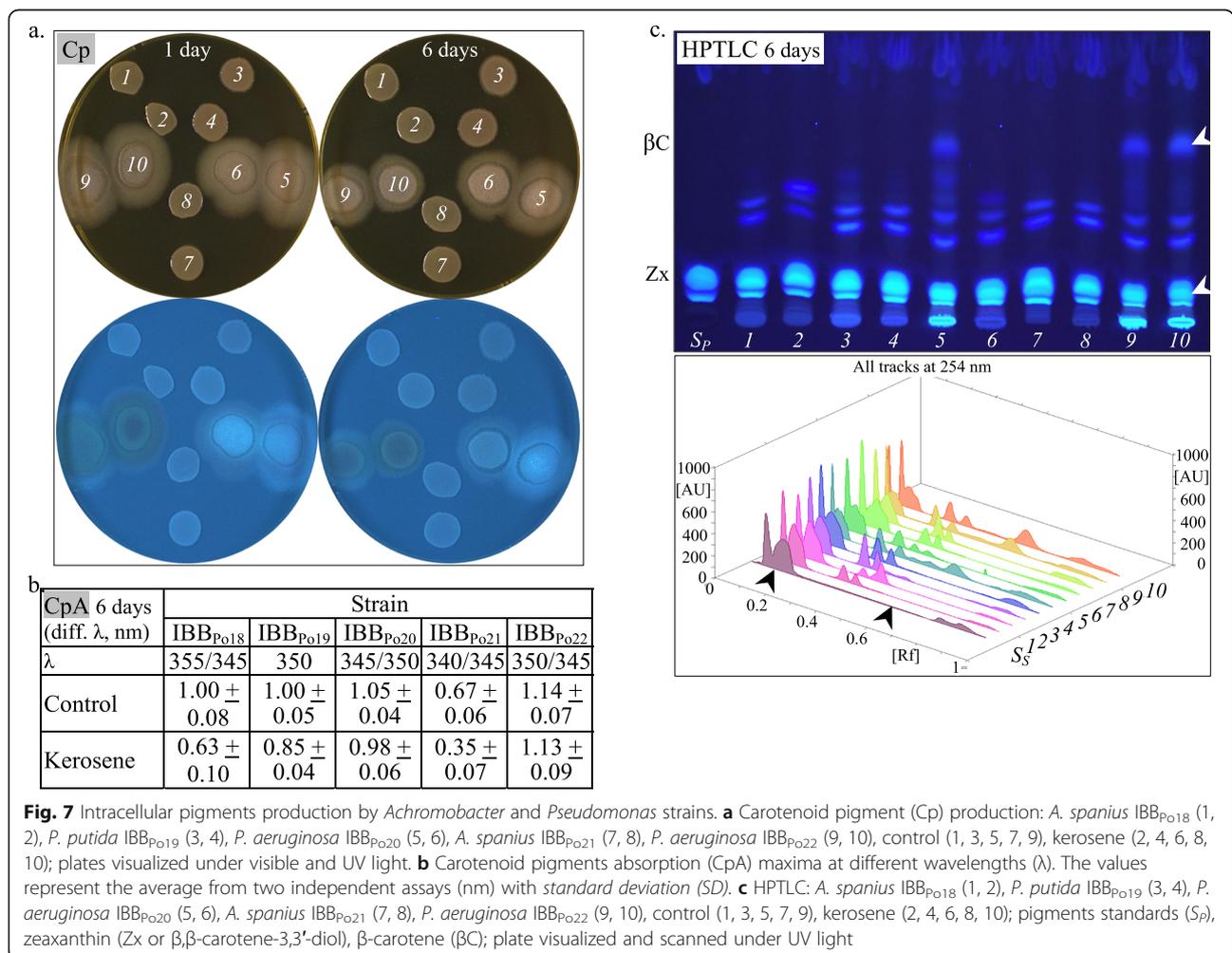
The ability of the isolated bacteria to produce extracellular pigments, such as pyocyanin and pyoverdine pigments, was confirmed by determining the formation of blue-green colonies on King A and yellow-green fluorescent colonies



**Fig. 6** Extracellular pigments production by *Achromobacter* and *Pseudomonas* strains. **a** Pyocyanin (Pc) production: *A. spanius* IBB<sub>Po18</sub> (1, 2), *P. putida* IBB<sub>Po19</sub> (3, 4), *P. aeruginosa* IBB<sub>Po20</sub> (5, 6), *A. spanius* IBB<sub>Po21</sub> (7, 8), *P. aeruginosa* IBB<sub>Po22</sub> (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 ( $\mu\text{g ml}^{-1}$ ) =  $\text{OD}_{520} \times 17.072$ , not detected (ND). The values represent the average from two independent assays ( $\mu\text{g ml}^{-1}$ ) with standard deviation (SD). **c** HPTLC: *A. spanius* IBB<sub>Po18</sub> (1, 2), *P. putida* IBB<sub>Po19</sub> (3, 4), *P. aeruginosa* IBB<sub>Po20</sub> (5, 6), *A. spanius* IBB<sub>Po21</sub> (7, 8), *P. aeruginosa* IBB<sub>Po22</sub> (9, 10), control (1, 3, 5, 7, 9), kerosene (2, 4, 6, 8, 10); pigments standards (*S<sub>P</sub>*), 2-heptyl-4-hydroxy-quinolone (HHQ), *Pseudomonas* quinolone signal (PQS); plate visualized and scanned under UV light

on King B agar, respectively. Although all tested bacteria were able to grow on these agar media, the pyocyanin (Fig. 6a) and/or pyoverdinin pigments production was observed only for *P. aeruginosa* isolates. *P. aeruginosa* IBB<sub>Po20</sub> produced both pyocyanin and pyoverdinin pigments, while *P. aeruginosa* IBB<sub>Po22</sub> 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* IBB<sub>Po20</sub> and IBB<sub>Po22</sub> showed characteristic peaks at 205–210, 275, 390, and 525 nm, and the pigment production significantly decreased from 32.40 to 0.30  $\mu\text{g ml}^{-1}$  and from 74.96 to 3.89  $\mu\text{g ml}^{-1}$ , 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* R<sub>1</sub>, *P. aeruginosa* U<sub>3</sub>) 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  $R_f$  values of 0.04–0.50 were observed in extracts from *A. spanius* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>, *P. putida* IBB<sub>Po19</sub>, and *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>, and the number of these fractions varies from one bacterium to another. The spot with  $R_f$  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* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>. 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



**Fig. 7** Intracellular pigments production by *Achromobacter* and *Pseudomonas* strains. **a** Carotenoid pigment (Cp) production: *A. spanius* IBB<sub>Po18</sub> (1, 2), *P. putida* IBB<sub>Po19</sub> (3, 4), *P. aeruginosa* IBB<sub>Po20</sub> (5, 6), *A. spanius* IBB<sub>Po21</sub> (7, 8), *P. aeruginosa* IBB<sub>Po22</sub> (9, 10), control (1, 3, 5, 7, 9), kerosene (2, 4, 6, 8, 10); plates visualized under visible and UV light. **b** Carotenoid pigments absorption (CpA) maxima at different wavelengths ( $\lambda$ ). The values represent the average from two independent assays (nm) with standard deviation (SD). **c** HPTLC: *A. spanius* IBB<sub>Po18</sub> (1, 2), *P. putida* IBB<sub>Po19</sub> (3, 4), *P. aeruginosa* IBB<sub>Po20</sub> (5, 6), *A. spanius* IBB<sub>Po21</sub> (7, 8), *P. aeruginosa* IBB<sub>Po22</sub> (9, 10), control (1, 3, 5, 7, 9), kerosene (2, 4, 6, 8, 10); pigments standards ( $S_p$ ), zeaxanthin (Zx or  $\beta,\beta$ -carotene-3,3'-diol),  $\beta$ -carotene ( $\beta\text{C}$ ); plate visualized and scanned under UV light

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* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>, *P. putida* IBB<sub>Po19</sub>, and *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub> (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* IBB<sub>Po18</sub> and IBB<sub>Po21</sub>, *P. putida* IBB<sub>Po19</sub>, and *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>, 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 ( $\beta$ C) 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 IBB<sub>Po18</sub> and IBB<sub>Po21</sub>, *P. putida* strain IBB<sub>Po19</sub>, and *P. aeruginosa* strains IBB<sub>Po20</sub> and IBB<sub>Po22</sub>. 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 pyoverdine pigments were produced only by *P. aeruginosa* IBB<sub>Po20</sub> and IBB<sub>Po22</sub>, 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

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