The tolerance of gram-negative bacterial strain to saturated and aromatic hydrocarbons

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MIHAELA LĂZĂROAIE*
Center of Microbiology, Institute of Biology, 296 Spl. Independentei St, 060031, PO 56-53, Bucharest, Romania, Phone 0040-21-2239072. Fax 0040-21-2219071;
e-mail: mihaela.lazaroaie@ibiol.ro

Abstract

The gram-negative Ralstonia pickettii IBB_1, Chryseobacterium indologenes IBB_2, Pseudomonas fluorescens IBB_3, Pseudomonas aeruginosa IBB_4, Burkholderia cepacia IBB_5 bacterial strains were able to tolerance high concentration of saturated (n-hexane, n-hexadecane, cyclohexane) and aromatic (benzene, toluene, ethylbenzene, xylene, 2-methylnaphtalene) hydrocarbons. The tolerance of gram-negative bacterial strains to saturated and aromatic hydrocarbons differed from one strain to another, and even for the same bacterial strain according to the nature of the hydrophobic substrate, and to the hydrocarbons concentration (5-15% v/v), with values between 0.004 and 1.651. The adhesion of gram-negative bacterial cells to saturated and aromatic hydrocarbons depended on the substrate nature, which is in direct correlation with the solubility in water and its hydrophobicity, with values between 0.25% and 54.11%. The presence in culture media of 10% (v/v) saturated and aromatic hydrocarbons induced modifications on cellular and molecular level to the strain Pseudomonas aeruginosa IBB_4: decrease of cells viability, modification of pigment production (pyocyanine), modifications on membranar and periplasmic protein profile (induction or repression of the synthesis of some proteins) and modification on lipid profile (elevated levels of phosphatidylglycerol, phosphatidylethanolamine or absence of phosphatidylethanolamine, phosphatidylglycerol, cardiolipin).

Keywords: bacteria, gram-negative, hydrocarbons, tolerance, modifications.

Introduction

Great efforts are carried out in order to develop strategies and technologies for preventing and controlling global pollution with hydrocarbons. Still, the increasing numbers of accidents that occur in marine and terrestrial environments make the pollution with hydrocarbons a major problem for the next decades. Bacteria are the most important microorganisms involved in natural decomposition of hydrocarbons.

According to the literature [1-3], there is an increasing interest in culturing of bacteria in two-liquid-phase systems consisting of an aqueous medium and hydrocarbons. This culture system is advantageous for bioconversion of hydrocarbons with low solubility in water. Hydrocarbons used in these systems are toxic for microorganisms because hydrocarbons molecules integrate into biological membranes and disturb the structure and function of the cell membrane [4-17]. Microbial cells' sensitivity to hydrocarbons depends on the logarithm of the partition coefficient of the hydrocarbon in octanol-water mixture (log P_{OW}). It has been stated that the hydrocarbons toxicity is generally in inverse correlation with log P_{OW} [2, 18]. This empirical log P_{OW} toxicity rule is based upon the fact that hydrocarbons with lower log P_{OW} (1.5-3.5) values bind more abundantly to viable bacterial cells, and hydrocarbons with higher log P_{OW} (>3.5) values bind less abundantly to viable bacterial cells [6].

Although different hydrocarbons are toxic for most microorganisms, there are bacterial strains able to tolerate high concentrations of such compounds in their growth medium. Gram-negative bacteria are less sensitive to hydrocarbons than gram-positive bacteria [19]. The biotechnological importance of gram-negative bacteria makes them ideal candidates for cellular and molecular studies. Tolerance and adhesion of gram-negative bacteria to 5-15% (v/v) saturated (n-hexane, n-hexadecane, cyclohexane) and aromatic (benzene, toluene, ethylbenzene, xylene, 2-methylnaphtalene) hydrocarbons were tested in this study. The modifications induced on cellular and molecular level on the *Pseudomonas aeruginosa* IBB_4, grown in the presence of 10% (v/v) saturated and aromatic hydrocarbons, are also presented in this study.

Materials and Methods

Bacterial strains. The hydrocarbon-degrading bacterial strains were isolated from aquatic and terrestrial sites, polluted with petroleum and petroleum products. The isolation of the hydrocarbon-degrading bacterial strains was done on mineral medium, with crude oil 5% (v/v) as single carbon source. The taxonomic affiliation of bacterial strains was established based on their phenotypic characteristics: Gram reaction, morphology, endospore formation, mobility, respirator type, pigments production, growth on TTC medium, catalase production, oxidase production, nitrates reduction, indole production, D-glucose fermentation, L-arginine dihydrolase and urease production, escualin and gelatin hydrolysis, β-galactosidase production, and assimilation of different substrates (bioMérieux API 20 NE kit).

Culture conditions. The bacteria were grown aerobically at 28°C in liquid LB-Mg medium consisting of 1% Bacto Tryptone (Difco), 0.5% Bacto Yeast Extract (Difco), 1% NaCl, with or without 10 mM $MgSO_4$. When necessary, the liquid LB-Mg medium was solidified with 1.5% (w/v) agar.

1. **Tolerance of gram-negative bacteria to saturated** (n-hexane, n-hexadecane, cyclohexane) and **aromatic** (benzene, toluene, ethylbenzene, xylene, 2-methylnaphtalene) **hydrocarbons**.
   - Bacterial cells were cultivated on **liquid** LB-Mg medium (control) and on LB-Mg medium in the presence of 5-15% (v/v) hydrocarbons. The growth of the bacterial strain was determined by spetrophotometric measurement of the optical density (OD_{660}) after 24 hours incubation at 28°C.
   - Bacterial cells in the exponential phase of growth (10^6 cells/ml) were plated on **solid** LB-Mg medium (control) and on LB-Mg medium that was overlaid with hydrocarbons. The formation of hydrocarbon-resistant bacterial colonies on the agar was estimated after 24 hours incubation at 28°C.
2. **Isolation, purification and visualization of total DNA of gram-negative bacteria**. The bacterial cells were lysed with TE buffer (10 mM Tris-HCl, 1 mM EDTA) and the genomic DNA was extracted with phenol-chloroform isoamilic alcohol (25:24:1), precipitated with ethanol and resuspended in TE buffer. After separation on 0.8% (w/v) TBE agarose gel and ethidium bromide staining (Tris-Borate-EDTA) the genomic DNA was visualized with ultraviolet. DNA content and purity was measured by the method of Sambrook et al. [20].
3. **Adhesion of gram-negative bacterial cells to saturated** (n-hexane, n-hexadecane, cyclohexane) and **aromatic** (benzene, toluene, ethylbenzene, xylene, 2-methylnaphtalene) **hydrocarbons.** The bacterial adhesion to hydrocarbons was determined using the method of Rosenberg et al. [21]. Bacterial cells suspension with optical density (OD_{660}) at 0.400 (A_1) was mixed with 5%, 10%, 15% (v/v) hydrocarbons. The tubes were stirred for 2 minutes and left at the room temperature for 15 minutes. It was determined OD_{660} of the aqueous layer for each sample (A_{2S}) and the OD_{660} for the control sample (A_{2C}). The bacterial adhesion to different hydrocarbons was calculated in percents with the formula: BATH (%) = $[(A_{2C} - A_{2S}) / A_1] \times 100$.
4. **Modifications induced on cellular and molecular level by saturated** (n-hexane, n-hexadecane, cyclohexane) and **aromatic** (benzene, toluene, ethylbenzene, xylene, 2-methylnaphtalene) **hydrocarbons on the** *Pseudomonas aeruginosa* IBB_4 **bacterial strain.** Cells were cultivated on liquid LB-Mg medium (control) and on LB-Mg medium in the presence of 10% (v/v) hydrocarbons. The modifications induced by hydrocarbons were determined after 48 hours incubation at 28°C.
   - **4.1** ***Modifications induced to cells viability and to pigment production.*** The bacterial cells, harvested by centrifugation, were washed in 0.86% (w/v) saline solution and resuspended in the same solution. The serial dilutions were spread on LB-Mg agar medium and there was determined the number of viable cells (CFU/ml). The wet mount was examined in an epifluorescence microscope and it was determined the modification on pigment production.
   - **4.*2 Modifications induced to proteins.*** The bacterial cells were resuspended in 10mM HEPES-NaOH, pH 7.6, which contains 10mM EDTA, and 10mM $MgCl_2$. Before the polyacrylamide gel electrophoresis, the samples were solved in Laemmli buffer (consisting of 25% glycerol, 14.4 mM β-mercaptoethanol, 2% sodium dodecyl sulfate, 60 mM Tris-HCl pH 6.8, and 0.1% bromophenol blue) and denaturated at 95°C, for 5 min. Gels were run for 3 h at 100 V through a 5% (w/v) acrylamide stacking gel and 10% (w/v) acrylamide separating gel. Gels were stained with Coomassie brilliant blue and destained in ethanol-glacial acetic acid-water (4.5:1:4.5 v/v/v). Protein content was measured by the method of Bradford [22]. Bovine serum albumin was used as the standard.
   - **4.*3 Modifications induced to lipids.*** The lipids were extracted by a modified Bligh-Dyer technique [23]. The samples were spotted onto 20×20cm Silica gel 60 TLC aluminium sheets (Merck), and the separation was performed using a mixture of chloroform-methanol-acetic acid-water (85:22.5:10:4 v/v/v/v) as mobile phase. For phospholipids visualization, the plates were treated with 10% (w/v) molybdatophosphoric acid hydrate in ethanol, and after drying the spots appeared on green background. The identification of the investigated components was done based on their motilities (R_f) and their comparison with those of phospholipid standards.

Reagents. n-hexane (96% pure), n-hexadecane (99% pure), cyclohexane (99.7% pure), benzene (99% pure), toluene (99% pure), xylene (97% pure) were procured from Merck. Ethylbenzene (98% pure), 2-methylnaphtalene (97% pure) were procured from Sigma-Aldrich. Other reagents used were procured from Merck (E. Merck, Darmstadt, Germany), Sigma-Aldrich (Saint-Quentin-Fallavier, France), Difco Laboratories, (Detroit, Michigan, USA), Promega (Promega GmbH, Mannheim, Germany) or bioMérieux (Marcy-l'Etoile, France).

Results and Discussions

The gram-negative bacterial strains were isolated using the enriched cultures method, which is considered as a very efficient method for the isolation and selection of the hydrocarbon-degrading bacterial strains. The use of mineral medium with hydrocarbons as single carbon source allows the selective development of the hydrocarbon-degrading bacterial strains. The taxonomic affiliation of bacterial strains was established based on their phenotypic characteristics (Table 1) and according with the API identification database (version 6). The

identification result for strain IBB_1 with the profile 0240575 corresponded to *Ralstonia pickettii* (good identification score), IBB_2 with the profile 2410004 corresponded to *Chryseobacterium indologenes* (good identification score), IBB_3 with the profile 1147475 corresponded to *Pseudomonas fluorescens* (very good identification score), IBB_4 with the profile 1554575 corresponded to *Pseudomonas aeruginosa* (good identification score), IBB_5 with the profile 5476777 corresponded to *Burkholderia cepacia* (good identification score).

Table 1. Phenotypic characteristics of bacterial strains

Characteristics	Bacterial strains:				
	IBB_1	IBB_2	IBB_3	IBB_4	IBB_5
Gram reaction	-	-	-	-	-
Morphology	singly / in pairs rods	singly / in pairs rods	singly / in pairs rods	singly / in pairs rods	singly rods
Endospore formation	-	-	-	-	-
Mobility	+	+	+	+	+
Respirator type	A	A	A	A	A
Pigment production on LB	white	yellow-orange	yellow-fluorescent	blue-green	red-brown
Pigment production on King A	-	-	-	+	-
Pigment production on King B	-	-	+	+	-
Growth on TTC medium	-	-	-	+	-
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Nitrates reduction	-	-	+	+	+
Indole production	-	+	-	-	-
D-glucose fermentation	-	-	-	-	+
L-arginine dihydrolase	-	-	+	+	-
Urease	+	-	-	-	-
Escualin 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	-	-	-	-	+

Legend: A = aerobic.

The gram-negative bacterial strains *Ralstonia pickettii* IBB_1, *Chryseobacterium indologenes* IBB_2, *Pseudomonas fluorescens* IBB_3, *Pseudomonas aeruginosa* IBB_4, *Burkholderia cepacia* IBB_5 were able to grow on liquid (Table 2) and solid (Table 3) LB-Mg medium, in the presence of saturated (n-hexane, n-hexadecane, cyclohexane) and aromatic (benzene, toluene, ethylbenzene, xylene, 2-methylnaphtalene) hydrocarbons.

The intensity of bacterial growth in the liquid medium, estimated through the degree of turbidity of the culture liquids (OD_{660}), differs from one strain to another, and even for the same bacterial strain according to the nature of the hydrophobic substrate, and to the hydrocarbons concentration (5-15% v/v), with values between 0.004 and 1.651 (Table 2). Generally, OD was higher for control compared with OD for variants added with saturated or aromatic hydrocarbons. However, when the strains *Ralstonia pickettii* IBB_1, *Chryseobacterium indologenes* IBB_2, *Pseudomonas aeruginosa* IBB_4 and *Burkholderia cepacia* IBB_5 were cultivated in the presence of 5% (v/v) n-hexadecane OD were higher than OD for each control. Considering that the tested bacterial strains are hydrocarbon-degrading strains, it is possible that the growth of these strains in the presence of some hydrocarbons could be enhanced by these hydrocarbons.

Table 2. Gram-negative bacterial strains tolerance to saturated and aromatic hydrocarbons on liquid medium

Variant	log P_{OW}[a]	Hb. conc. (v/v)[b]	Bacterial strains growth (OD_{660}) on liquid medium:				
			R. pickettii IBB_1	*C. indologenes* IBB_2	*P. fluorescens* IBB_3	*P. aeruginosa* IBB_4	*B. cepacia* IBB_5
LB-Mg (Control)	-	-	1.590	1.273	1.085	1.598	1.429
LB-Mg + n-Hexane	3.86	5%	1.338	1.127	0.637	1.265	1.356
		10%	0.817	1.048	0.444	1.013	1.212
		15%	0.514	0.531	0.036	0.892	1.068

LB-Mg + n-Hexadecane	9.15	5%	1.651	1.302	0.758	1.617	1.476
		10%	1.557	1.176	0.544	1.523	1.324
		15%	1.409	1.017	0.419	1.431	1.078
LB-Mg + Cyclohexane	3.35	5%	0.943	0.943	0.526	1.352	1.242
		10%	0.771	0.484	0.431	0.756	1.037
		15%	0.609	0.205	0.056	0.584	0.610
LB-Mg + Benzene	2.14	5%	0.031	0.051	0.531	1.414	1.010
		10%	0.007	0.013	0.031	0.703	0.077
		15%	0.007	0.004	0.024	0.008	0.026
LB-Mg + Toluene	2.64	5%	1.256	0.248	0.892	0.392	0.034
		10%	0.423	0.022	0.712	0.222	0.025
		15%	0.033	0.004	0.403	0.004	0.009
LB-Mg + Xylene	3.14	5%	1.297	0.898	0.714	1.261	0.685
		10%	1.075	0.636	0.579	1.100	0.079
		15%	0.987	0.205	0.470	0.772	0.026
LB-Mg + Ethylbenzene	3.17	5%	0.927	0.849	0.779	1.446	0.474
		10%	0.882	0.316	0.627	1.066	0.335
		15%	0.784	0.216	0.370	0.882	0.056
LB-Mg + 2-Methylnaphtalene	3.81	5%	0.954	0.855	1.148	1.083	0.759
		10%	0.593	0.667	1.063	0.942	0.563
		15%	0.470	0.591	0.655	0.774	0.311

Legend: ^{a} = logarithm of the partition coefficient of the hydrocarbon in octanol-water mixture (log P_{OW});

^{b} = hydrocarbon concentration (v/v).

I have already mentioned that microbial cells' sensitivity to hydrocarbons depends on the logarithm of the partition coefficient of the hydrocarbon in octanol-water mixture (log P_{OW}). Benzene, toluene, xylene, ethylbenzene and cyclohexane, with log P_{OW} between 2.14 and 3.35, are more toxic for tested gram-negative bacterial strains, compared with 2-methylnaphtalene, n-hexane and n-hexadecane, with log P_{OW} between 3.81 and 9.15. However, each bacterium has its own intrinsic level of tolerance to hydrocarbons. The increase of saturated and aromatic hydrocarbon concentration leads to a decrease of the optical density values (Table 2). Tsukagoshi et al. [2] considered that certain bacterial cells accumulate hydrocarbons in a manner positively dependent on the log P_{OW} and the concentration of the hydrocarbons in the medium.

Ralstonia pickettii IBB_1 presented very good tolerance (OD = 0.514-1.651) to 5%, 10%, 15% (v/v) n-hexane, n-hexadecane, cyclohexane, xylene, ethylbenzene, 5%, 10% (v/v) 2-methylnaphtalene, 5% (v/v) toluene, good tolerance (OD = 0.423, 0.470) to 10% (v/v) toluene, 15% (v/v) 2-methylnaphtalene, and high sensitivity (OD = 0.007-0.033) to 5%, 10%, 15% (v/v) benzene (maybe for benzene small concentrations are necessary and bacterial strain adhesion should be considered), 15% (v/v) toluene.

Chryseobacterium indologenes IBB_2 presented very good tolerance (OD = 0.531-1.302) to 5%, 10%, 15% (v/v) n-hexane, n-hexadecane, 2-methylnaphtalene, 5%, 10% (v/v) xylene, 5% (v/v) cyclohexane, ethylbenzene, good tolerance (OD = 0.205-0.484) to 5% (v/v) toluene, 10%, 15% (v/v) cyclohexane, ethylbenzene, 15% (v/v) xylene, and high sensitivity (OD = 0.004-0.051) to 5%, 10%, 15% (v/v) benzene, 10%, 15% (v/v) toluene.

Pseudomonas fluorescens IBB_3 presented very good tolerance (OD = 0.526-1.148) to 5%, 10%, 15% (v/v) 2-methylnaphtalene, 5%, 10% (v/v) n-hexadecane, toluene, xylene, ethylbenzene, 5% (v/v) n-hexane, cyclohexane, benzene, good tolerance (OD = 0.370-0.470) to 10% (v/v) n-hexane, cyclohexane, 15% (v/v) n-hexadecane, toluene, xylene, ethylbenzene, and high sensitivity (OD = 0.024-0.056) to 15% n-hexane, cyclohexane, 10%, 15% (v/v) benzene.

Pseudomonas aeruginosa IBB_4 presented very good tolerance (OD = 0.584-1.617) to 5%, 10%, 15% (v/v) n-hexane, n-hexadecane, cyclohexane, xylene, ethylbenzene, 2-methylnaphtalene, 5%, 10% (v/v) benzene, good tolerance (OD = 0.392, 0.222) to 5%, 10% (v/v) toluene, and high sensitivity (OD = 0.004, 0.008) to 15% (v/v) toluene, benzene.

Burkholderia cepacia IBB_5 presented very good tolerance (OD = 0.563-1.476) to 5%, 10%, 15% (v/v) n-hexane, n-hexadecane, cyclohexane, 5%, 10% (v/v) 2-methylnaphtalene, 5% (v/v) benzene, xylene, good tolerance (OD = 0.311-0.474) to 5%, 10% ethylbenzene, 15% (v/v) 2-methylnaphtalene, and high sensitivity (OD = 0.009-0.079) to 5%, 10%, 15% (v/v) toluene, 10%, 15% (v/v) benzene, xylene, 15% (v/v) ethylbenzene.

The growth of gram-negative bacterial strains on agar LB-Mg medium overlaid with hydrocarbons was 25-100% in the presence of saturated hydrocarbons, and 10-75% in the presence of aromatic hydrocarbons (Table 3). *Ralstonia pickettii* IBB_1 presented high sensitivity (0.01%) in the presence of benzene, *Chryseobacterium indologenes* IBB_2 presented high sensitivity (0.01%) in the presence of cyclohexane, benzene, and *Pseudomonas fluorescens* IBB_3, *Pseudomonas aeruginosa* IBB_4 presented high sensitivity (0.01%) in the presence of n-hexane, benzene. *Burkholderia cepacia* IBB_5 wasn't sensitive to the presence on the surface of agar medium of saturated and aromatic hydrocarbons, so that the bacterial growth was 25-100%.

Table 3. Gram-negative bacterial strains tolerance to saturated and aromatic hydrocarbons on solid medium

Variant	log P_{OW}[a]	Bacterial strains growth on solid medium[b]:				
		R. pickettii IBB_1	*C. indologenes* IBB_2	*P. fluorescens* IBB_3	*P. aeruginosa* IBB_4	*B. cepacia* IBB_5
LB-Mg (Control)	-	100%	100%	100%	100%	100%
LB-Mg + n-Hexane	3.86	100%	100%	0.01%	0.01%	100%
LB-Mg + n-Hexadecane	9.15	100%	100%	100%	100%	100%
LB-Mg + Cyclohexane	3.35	25%	0.01%	100%	100%	100%
LB-Mg + Benzene	2.14	0.01%	0.01%	0.01%	0.01%	25%
LB-Mg + Toluene	2.64	10%	75%	25%	50%	25%
LB-Mg + Xylene	3.14	10%	75%	25%	25%	25%
LB-Mg + Ethylbenzene	3.17	10%	75%	25%	50%	25%
LB-Mg + 2-methylnaphtalene	3.81	25%	75%	50%	25%	75%

Legend: [a] = logarithm of the partition coefficient of the hydrocarbon in octanol-water mixture (log P_{OW}).

[b] = the hydrocarbon resistance is represented by the frequency of colony formation, with that observed in the absence of any hydrocarbon taken as 100%.

For three bacterial strains it was observed pigment production modifications when the growth was done on solid medium in the presence of aromatic hydrocarbons. *Pseudomonas fluorescens* IBB_3 has lost pyoverdine/fluoresceine, *Pseudomonas aeruginosa* IBB_4 has lost pyocyanine, and *Burkholderia cepacia* IBB_5 has lost melanin pigment.

Pseudomonas fluorescens IBB_3, *Pseudomonas aeruginosa* IBB_4, *Burkholderia cepacia* IBB_5 have chromosomal and plasmid DNA, while *Ralstonia pickettii* IBB_1, *Chryseobacterium indologenes* IBB_2 have only chromosomal DNA. The high tolerance to hydrocarbons of *Pseudomonas fluorescens* IBB_3, *Pseudomonas aeruginosa* IBB_4, *Burkholderia cepacia* IBB_5 is caused by the presence of some hydrocarbon resistance plasmid, and the high tolerance to hydrocarbons of *Ralstonia pickettii* IBB_1, *Chryseobacterium indologenes* IBB_2 is caused by the presence of a hydrocarbon resistance factor, located on the chromosome.

Although the hydrocarbons are compounds with relatively low water solubility, the solubility rate may increase by increasing their specific surface, as a result of the mechanical dispersion realized by stirring of the tubes containing the aqueous phase (cell suspension) and the organic phase (hydrocarbon). The adhesion of cells to hydrocarbons depends on the substrate nature, which is in direct correlation with the solubility in water and its hydrofobicity. The hydrocarbon quantities bound by bacterial cells differ from one bacterial strain to another, with values between 0.25% and 54.11% (Table 4).

The increase of saturated and aromatic hydrocarbon concentration led to a decrease of the bacterial adhesion values. There were significant differences between bacterial adhesion obtained when the hydrocarbons were used in concentration of 5% (v/v), compared with bacterial adhesion to the same hydrocarbons, when hydrophobic substrates were used in concentration of 10% and especially of 15% (v/v), due to some structural modifications at the cell envelope level. The low hydrophobicity of the cell envelope represents a defensive mechanism, which prevents the hydrocarbons accumulation in the bacterial cell membranes. Therefore, the cell envelope modification from hydrophobic to hydrophilic, by increasing the organization of the lipid bilayer, by increasing the number of cross-linking between cell wall components or by modification of the lipopolysaccharides of the outer membrane of the gram-negative bacteria, is a protection mean of the bacterial cells against the high concentrations of hydrocarbons [6, 24, 25].

Table 4. Gram-negative bacterial strains adhesion to saturated and aromatic hydrocarbons

Variant	Hb. conc. (v/v)[a]	The decrease of the turbidity in aqueous phase (%):				
		R. pickettii IBB_1	*C. indologenes* IBB_2	*P. fluorescens* IBB_3	*P. aeruginosa* IBB_4	*B. cepacia* IBB_5
n-Hexane	5%	46.38	29.48	14.86	11.17	8.04
	10%	43.89	28.50	4.53	5.32	7.54
	15%	32.67	13.27	3.27	3.19	6.28
n-Hexadecane	5%	47.63	21.13	10.58	37.77	9.05
	10%	44.89	12.04	8.06	29.26	5.03
	15%	31.67	5.41	0.50	22.34	0
Cyclohexane	5%	45.89	2.46	5.54	9.31	3.77
	10%	39.15	1.72	4.53	3.72	3.52
	15%	33.42	0	3.53	0	0
Benzene	5%	27.43	4.91	35.01	32.71	0
	10%	26.68	4.67	26.70	11.44	0
	15%	26.43	3.44	24.18	9.84	0
Toluene	5%	29.68	0.74	9.57	23.14	0.75
	10%	25.19	0.25	6.30	19.15	0.25
	15%	12.72	0	2.52	14.63	0
Xylene	5%	54.11	1.97	19.65	31.38	5.53
	10%	35.66	1.47	11.34	21.28	5.28
	15%	33.17	1.23	0.25	20.48	2.76
Ethylbenzene	5%	31.42	0.49	1.51	0.53	1.51
	10%	22.69	0.25	1.01	0.27	0.75
	15%	2.74	0	0.76	0	0.50
2-Methylnaphtalene	5%	0	0	0	0	0

	10%	0	0	0	0	0
	15%	0	0	0	0	0

Legend: ^{a} = hydrocarbon concentration (v/v).

I chose the strain *Pseudomonas aeruginosa* IBB_4 for the study of cellular and molecular modifications induced by hydrocarbons because my previous results showed that this strain was capable to tolerate high concentrations of saturated and aromatic hydrocarbons (5%, 10% and sometime 15% v/v) in liquid culture media.

According to the literature [3, 5, 7-10, 12, 25-30] hydrocarbons are generally toxic to microbial cells, as a result of their partition in the lipid bilayer, where they can cause significant modifications in the membrane structure and functions (loss of ions, metabolites, lipids, proteins; dissipation of the pH gradient and electrical potential; inhibition of membrane proteins functions). This is often followed by cell lysis and death.

The *Pseudomonas aeruginosa* IBB_4 cells presented a higher viability (3.8×10^{14} CFU/ml) when growth was done in the absence of hydrocarbons (control), compared with the viability of the bacterial cells grown in the presence of 10% (v/v) saturated and aromatic hydrocarbons (1.0×10^{11} - 3.6×10^{13} CFU/ml) (Fig. 1).

There were no modifications of the pigment production (pyocyanine) of the *Pseudomonas aeruginosa* IBB_4 cells, cultivated on liquid medium in the absence of hydrocarbons (control) or in the presence of 10% (v/v) n-hexane, n-hexadecane. But, it were observed modifications of pyocianine production of the *Pseudomonas aeruginosa* IBB_4 cells, cultivated on liquid medium in the presence of 10% (v/v) cyclohexane or aromatic hydrocarbons, fact confirmed by the epifluorescence microscope observations (Fig. 1).

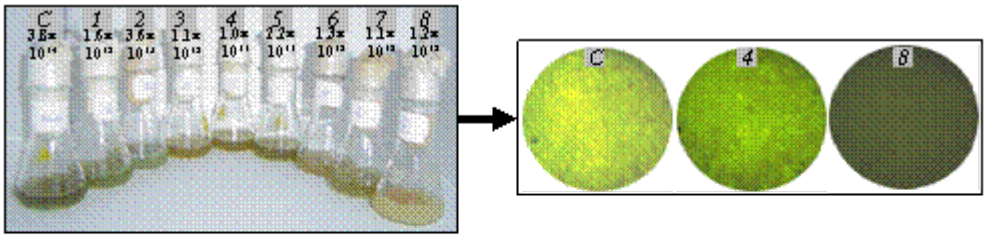

Figure 1. Modifications induced by 10% (v/v) saturated and aromatic hydrocarbons on cells viability and pigment production of *Pseudomonas aeruginosa* IBB_4

(*C*) Control; (*1*) n-hexane, (*2*) n-hexadecane; (*3*) cyclohexane; (*4*) benzene; (*5*) toluene; (*6*) xylene; (*7*) ethylbenzene; (*8*) 2-methylnaphtalene.

The electrophoresis studies in polyacrilamide gel showed the existence of some differences in the membranar and periplasmic protein profile of the *Pseudomonas aeruginosa* IBB_4 cells grown in the absence of hydrocarbons (control), compared with the protein profile of the cells grown in the presence of 10% (v/v) saturated and aromatic hydrocarbons, except n-hexadecane, toluene, and 2-methylnaphtalene (Fig. 2*A*). In the presence of 10% (v/v) n-hexane, cyclohexane, xylene, ethylbenzene it was observed the induction of the synthesis of some proteins (50-, 42-, 37-, 30-, 28-kDa), while in the presence of 10% (v/v) benzene it was observed the repression of the synthesis of some proteins (187-, 105-, 87-, 68-, 58-, 50-, 42-, 40-, 37-, 30-, 28-, 15-, 10-kDa).

Thin-layer chromatography studies showed the existence of some modification in lipid profile of the *Pseudomonas aeruginosa* IBB_4 cells grown in the absence of hydrocarbons (control), compared with the lipid profile of the cells grown in the presence of 10% (v/v) hydrocarbons (Fig. 2*B*). The phospholipids found, based on their R_f values, in *Pseudomonas aeruginosa* IBB_4 cells grown in the absence of hydrocarbons and in the presence of 10% (v/v) hydrocarbons were phosphatidylethanolamine (PE with R_f 0.57-0.65), phosphatidylglycerol (PG with R_f 0.85-0.86), and cardiolipin / diphosphatidylglycerol (CL with R_f 0.95). It was not observed the presence of phosphatidylcholine (PC) and phosphatidylserine (PS). *Pseudomonas aeruginosa*

IBB_4 cells grown in the presence of 10% (v/v) n-hexadecane, ethylbenzene, 2-methylnaphtalene, cyclohexane, benzene, and toluene had elevated levels of PG and PE. These changes are explained as a way for bacteria to maintain membrane fluidity and impermeability [5, 10, 14]. *Pseudomonas aeruginosa* IBB_4 cells grown in the presence of 10% (v/v) xylene exhibit the absence of PE, PG and CL. This was expected, because the electron microscopy studies made evident the appearance of some lesions at the outer membrane level (data not shown).

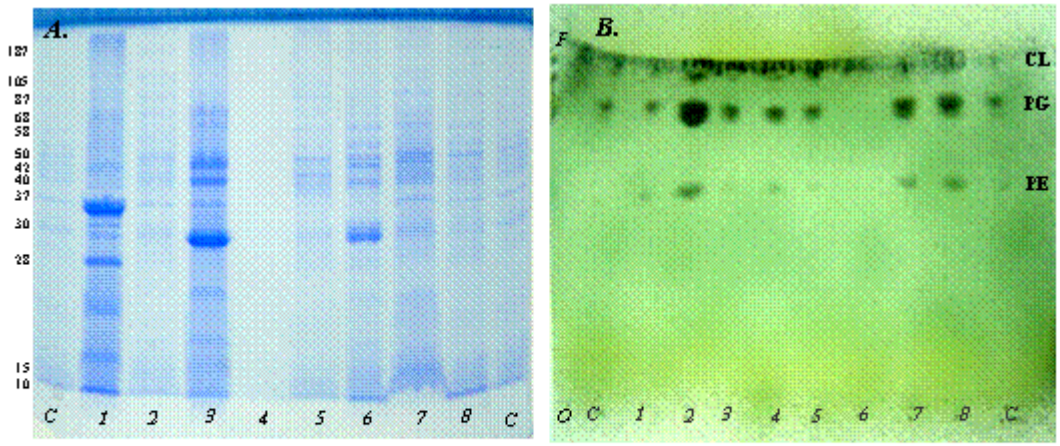

Figure 2. Modifications induced by 10% (v/v) saturated and aromatic hydrocarbons on protein (*A*) and lipid (*B*) profile of *Pseudomonas aeruginosa* IBB_4

(***C***) Control; (***1***) n-hexane, (***2***) n-hexadecane; (***3***) cyclohexane; (***4***) benzene; (***5***) toluene; (***6***) xylene; (***7***) ethylbenzene; (***8***) 2-methylnaphtalene; (***O***) origin; (***F***) solvent front.

Conclusions

The increased interest given to the researches on gram-negative bacteria isolated from environments highly contaminated with hydrocarbons is due to their high biotechnological potential, and also to the perspective of their use in different technologies for cleaning the environments polluted with such compounds. The tolerance and adhesion of bacterial strains to hydrocarbons has a great importance in hydrocarbons contaminated environments and they frequently influence the rate and efficiency of biodegradation.

Tolerance of gram-negative bacterial strains belonging to *Ralstonia*, *Chryseobacterium*, *Pseudomonas* and *Burkholderia* genera to saturated and aromatic hydrocarbons differs from one strain to another and even for the same bacterial strain according to the nature and concentration of the hydrophobic substrate. Benzene, toluene, xylene, ethylbenzene and cyclohexane, with log P_{OW} between 2.14 and 3.35, are more toxic for tested gram-negative bacterial strains, compared with 2-methylnaphtalene, n-hexane and n-hexadecane, with log P_{OW} between 3.81 and 9.15. However, each bacterium has its own intrinsic level of tolerance to hydrocarbons.

The adhesion of gram-negative bacterial cells to saturated and aromatic hydrocarbons depends on the substrate nature, which is in direct correlation with the solubility in water and its hydrophobicity, with values between 0.25% and 54.11%.

The presence in culture media of saturated and aromatic hydrocarbons induced cellular and molecular modifications to the strain *Pseudomonas aeruginosa* IBB_4. Saturated and aromatic hydrocarbons determined the decrease of cells viability. Cyclohexane and aromatic hydrocarbons determined modifications of pigment production (pyocyanine). Hexane, cyclohexane, benzene, xylene and ethylbenzene induced modification of membranar and periplasmic protein profile. Hexadecane, cyclohexane and aromatic hydrocarbons induced modification of lipid profile.

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