Full Paper

Multidrug resistance in hydrocarbon-tolerant Gram-positive and Gram-negative bacteria

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New Gram-positive and Gram-negative bacteria were isolated from Poeni oily sludge, using enrichment procedures. The six Gram-positive strains belong to *Bacillus*, *Lysinibacillus* and *Rhodococcus* genera. The eight Gram-negative strains belong to *Shewanella*, *Aeromonas*, *Pseudomonas* and *Klebsiella* genera. Isolated bacterial strains were tolerant to saturated (i.e., *n*-hexane, *n*-heptane, *n*-decane, *n*-pentadecane, *n*-hexadecane, cyclohexane), monoaromatic (i.e., benzene, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) and polyaromatic (i.e., naphthalene, 2-methylnaphthalene, fluorene) hydrocarbons, and also resistant to different antimicrobial agents (i.e., ampicillin, kanamycin, rhodamine 6G, crystal violet, malachite green, sodium dodecyl sulfate). The presence of hydrophilic antibiotics like ampicillin or kanamycin in liquid LB-Mg medium has no effects on Gram-positive and Gram-negative bacteria resistance to toxic compounds. The results indicated that Gram-negative bacteria are less sensitive to toxic compounds than Gram-positive bacteria, except one bacteria belonging to *Lysinibacillus* genus. There were observed cellular and molecular modifications induced by ampicillin or kanamycin to isolated bacterial strains. Gram-negative bacteria possessed between two and four catabolic genes (*alkB*, *alkM*, *alkB/alkB1*, *todC1*, *xyIM*, PAH dioxygenase, catechol 2,3-dioxygenase), compared with Gram-positive bacteria (except one bacteria belonging to *Bacillus* genus) which possessed one catabolic gene (*alkB/alkB1*). Transporter genes (*HAE1*, *acrAB*) were detected only in Gram-negative bacteria.

Key Words—bacteria; hydrocarbon-tolerant; multidrug resistance

Introduction

During evolutionary history, bacteria have been exposed to different toxic compounds, such as antibiotics, dyes, hydrocarbons, detergents, biocides, heavy metal ion, natural toxins, etc. To protect themselves, microorganisms have evolved mechanisms that de-

toxify and extrude these toxic compounds. This phenomenon leads to the occurrence of multidrug resistance (MDR), which is a unidirectional efflux system that catalyzes the active extrusion of a large number of structurally and functionally unrelated compounds (i.e., antibiotics, dyes, hydrocarbons, detergents, biocides, heavy metal ions) from the bacterial cytoplasm to the external medium (Borges-Walmsley et al., 2003; Ramos et al., 2002; Segura et al., 1999; Terán et al., 2003). The data available so far indicate that the physical characteristics of the compounds (charge, hydrophobicity or amphipathicity), rather than their structures, could be the determining factor in the specificities

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of these multidrug efflux systems (Segura et al., 1999). Efflux systems capable of transporting multiple antimicrobials fall into six superfamilies: (1) the major facilitator superfamily (MFS) (Pao et al., 1998; Poole, 2005); (2) the ATP-binding cassette (ABC) family (Poole, 2005); (3) the resistance-nodulation-division (RND) family (Nikaido, 1998; Poole, 2005); (4) the small multidrug resistance (SMR) family (which is itself a member of the drug-metabolite transporter superfamily) (Poole, 2005); (5) the multidrug and toxic compound extrusion (MATE) family (Brown et al., 1999; Poole, 2005); and (6) the multidrug endosomal transporter family (Poole, 2005; Terán et al., 2003). All of these transporters catalyze active efflux of antimicrobials and therefore require energy, mostly in the form of proton motif force or in the form of ATP (Putman et al., 2000; Schweizer, 2003). The members of the RND family are the most relevant in respect to resistance to different antimicrobial agents (Poole, 2001, 2005). Originally thought to be limited to Gram-negative bacteria, members of the RND family have now been identified in all major kingdoms (Brehm-Stecher and Johnson, 2003; Lin et al., 2002; Poole, 2001; Tseng et al., 1999; Zgurskaya and Nikaido, 2000). The efflux pumps work with exceptional efficiency in Gram-negative bacteria due to synergistic action of the cytoplasmic membrane with the outer membrane. In Gram-positive bacteria, the efflux pumps move the substrate across just one membrane. This is rather inefficient, as they have to compete with the rapid spontaneous influx of the lipophilic molecule back into the cytoplasm. A high rate of efflux is therefore required to produce significant levels of resistance. The efflux pumps in the Gram-negative bacteria traverse both the cytoplasmic and outer membranes (Nikaido, 1996, 1998, 2003; Zahir et al., 2006; Zgurskaya and Nikaido, 2000). As the outer membrane is composed largely of lipopolysaccharides (LPS), it has different permeability properties compared to the membrane of Gram-positive bacteria. The membrane of Gram-negative bacteria allows the penetration of lipophilic molecules but at a rate 20 to 100 times slower than the phospholipid bilayer of Gram-positive bacteria. The decrease in penetration of lipophilic molecules is responsible for the increased resistance of Gram-negatives to certain hydrophilic molecules. Hydrophilic molecules traverse the membrane of Gram-negative cells through porins. In the presence of hydrophilic molecules or when efflux mechanisms are induced, a decrease in the number of

porins in the membrane is also observed, leading to a decreased penetration of the hydrophilic molecules (Nikaido, 1998, 2003).

The present study focuses on the isolation and characterization of fourteen bacterial strains from Poeni oily sludge. The isolated bacteria were tolerant to saturated, monoaromatic and polyaromatic hydrocarbons and they were also resistant to hydrophilic antibiotics and other toxic compounds. Aono et al. (1995) were the first to establish a clear connection between hydrocarbon tolerance and antibiotic resistance. We investigated the effects of hydrophilic antibiotics, like ampicillin or kanamycin, on bacterial strain tolerance to saturated, monoaromatic and polyaromatic hydrocarbons. The modifications induced on the cellular and molecular level by ampicillin or kanamycin on isolated bacterial strains are also presented in this study. This is interesting because both ampicillin and kanamycin are hydrophilic drugs that are pumped out by the multidrug efflux systems also involved in hydrocarbon efflux. Furthermore, the bacterial strains were screened by PCR for the presence of some catabolic genes for hydrocarbon biodegradation and for some transporter genes important for toxic compound resistance.

Materials and Methods

Isolation and characterization of bacterial strains.

The number of viable bacteria in Poeni oily sludge (Teleorman County, Romania) was estimated by a modified most probable number (MPN) procedure (Haines et al., 1996). Aliquots of 20 μ l were added to ten separate dilution series in 96-microwell plates (Iwaki). The wells were pre-filled with 170 μ l minimal medium (Grifoll et al., 1995) and 10 μ l sterilized Poeni crude oil. After 2-weeks incubation at 28°C, each well was inspected for dispersion of crude oil ("Sheen Screen") and, subsequently received 50 μ l of filter-sterilized 0.3% (w/v) solution of the respiration indicator INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride]. The plates were incubated overnight in the dark, at room temperature and the red and pink wells were counted as positive for growth. A maximum-likelihood estimation of microbial numbers based on the 10-fold dilution series was calculated using the Microsoft Excel for Windows spreadsheet program developed by Garthright and Blodgett (2003).

Isolation of Po₁ to Po₁₄ bacterial strains from Poeni

oily sludge was carried out on minimal medium (Grifoll et al., 1995), using the enriched cultures method, with 1% (v/v) Poeni crude oil as the single carbon source. Bacterial strains were selected on the basis of different colony morphologies. The subsequent characterization of bacterial strains was performed, using several physiological and biochemical tests: Gram reaction, morphology, endospore formation, mobility, respirator type, pigment production, growth on TTC medium, and catalase and oxidase production. Gram-positive bacteria were investigated using an API Coryne kit (bioMérieux). Gram-negative bacteria were investigated using an API 20NE kit (bioMérieux). The taxonomic affiliation of isolated bacteria was determined on the basis of their phenotypic characteristics, on the G+C content of the bacterial chromosome (De Ley, 1976) and on their 16S rDNA sequence (Marchesi et al., 1998). Template DNA for PCR was obtained using the method of Whyte et al. (1996) and it was used to amplify an almost full-length 16S rDNA fragments. For PCR amplification, 1 μ l of DNA extract was added to a final volume of 25 μ l reaction mixture, containing 10 $\times$ PCR buffer with MgCl₂, dNTP mix, universal bacterial primers (27f, 1492r), *Taq* DNA polymerase (Biotools). PCR was performed with a Master Cycler Personal thermocycler (Eppendorf). PCR program consisted in initial denaturation for 10 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and an extension step at 72°C for 2 min, and a final extension at 72°C for 10 min. After separation on 1.2 (w/v) TBE agarose gel and staining with ethidium bromide the PCR products were visualized with ultraviolet light. The PCR products were then purified with a QIAEX II extraction kit (Qiagen). The nucleotide sequences of the purified PCR products were determined by using a BigDye terminator cycle sequencing kit (Applied Biosystems). The products of the sequencing reactions were analyzed with an automatic sequencer (ABI Prism 3700, Applied Biosystems). The BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) search program (BLASTN 2.2.23) was used to look for nucleotide sequence homology of these bacterial strains.

Resistance of isolated bacteria to hydrophilic antibiotics and toxic compounds. As hydrophilic antibiotics ampicillin and kanamycin were used. The toxic compounds tested were rhodamine 6G, crystal violet, malachite green, and sodium dodecyl sulfate. Bacterial cells were cultivated in liquid LB-Mg medium (Hei-

pieper et al., 1992) (control) and in the LB-Mg medium supplemented with 25 μ g ml⁻¹ ampicillin or kanamycin. All samples were incubated at 28°C on a rotary shaker (150–200 rpm). Bacterial cells (10⁵ CFU ml⁻¹) were then spotted (20 μ l) on solid LB-Mg medium (control) and on the solid LB-Mg medium supplemented with various concentrations of antibiotics and toxic compounds (5–1,000 μ g ml⁻¹). Petri dishes were incubated for 24 h at 28°C. MICs of antibiotics and toxic compounds were determined as the concentrations that severely inhibited bacterial cell growth. Rhodamine 6G accumulation in bacterial cells was observed under UV light after 24 h of incubation at 28°C. Here, as elsewhere in this work, experiments were repeated at least three times.

Effects of hydrophilic antibiotics on bacteria tolerance to saturated, monoaromatic, and polyaromatic hydrocarbons. The saturated hydrocarbons used were *n*-hexane, *n*-heptane, *n*-decane, *n*-pentadecane, *n*-hexadecane, and cyclohexane; the monoaromatic hydrocarbons used were benzene, toluene, styrene, xylene isomers, ethylbenzene, and propylbenzene; and the polyaromatic hydrocarbons used were naphthalene, 2-methylnaphthalene, and fluorene. Bacterial cells were cultivated in liquid LB-Mg medium (control) and in liquid LB-Mg medium supplemented with 25 μ g ml⁻¹ ampicillin or kanamycin. All samples were incubated at 28°C on a rotary shaker (150–200 rpm). Bacterial cells (10⁵ CFU ml⁻¹) were then spotted (20 μ l) on solid LB-Mg medium (control) and on the solid LB-Mg medium supplemented with hydrocarbons, supplied as a vapor phase. Petri dishes were sealed and the formation of hydrocarbon-resistant bacterial colonies on the agar was determined after 24 h incubation at 28°C.

Cellular and molecular modifications induced by hydrophilic antibiotics on isolated bacteria. Bacterial cells were cultivated in liquid LB-Mg medium (control) and in liquid LB-Mg medium supplemented with 25 μ g ml⁻¹ ampicillin or kanamycin. Flasks were incubated for 24 h at 28°C on a rotary shaker (150–200 rpm).

Modifications to cell viability induced by antibiotics. The growth of the bacterial strains was determined by spectrophotometric measurement of the optical density (OD_{660nm}). Serial dilutions of culture liquid were also spread on agar LB-Mg medium using the method of Ramos et al. (1997) and the number of viable cells (CFU ml⁻¹) was determined.

Effects of antibiotics on β -galactosidase activity.

Bacterial cells were permeabilized using chloroform and sodium dodecyl sulfate and assayed for β -galactosidase activity by measuring the hydrolysis of the chromogenic substrate, *o*-nitrophenyl- β -D-galactoside (Miller, 1972).

Modifications to lipids induced by antibiotics. Lipids were extracted with a chloroform-methanol (2 : 1) mixture using the method of Benning and Somerville (1992). The samples were spotted onto 20×20 cm Silica gel 60 TLC aluminium sheets (Merck), and the separation was performed using a chloroform-methanol-acetic acid-water (85 : 22.5 : 10 : 4, v/v/v/v) mixture as the mobile phase, in saturated atmosphere (simultaneous separating chamber of 21×9×21 cm, Desaga type). The plates were treated with 10% (w/v) molybdatophosphoric acid hydrate in ethanol, and after drying the spots appeared on a green background. The identification of the phospholipids was performed based on their *R_f* and their comparison with those of standard phospholipids (Sigma-Aldrich).

Modifications to proteins induced by antibiotics. Membrane and periplasmic protein fractions were extracted with HE buffer (10 mM HEPES-NaOH, pH 7.6, 10 mM EDTA, 10 mM MgCl₂), solved in Laemmli buffer and denaturated at 95°C, for 5 min. Fifty micrograms of

protein per lane were loaded onto a 12% (w/v) polyacrylamide gel (Sambrook et al., 1989). Gels were stained with Bio-Safe colloidal Coomassie Blue G-250 (Bio-Rad). Protein content was measured by the method of Bradford (1976).

Screening of isolated bacteria for the presence of catabolic genes for hydrocarbon biodegradation and transporter genes important for toxic compound resistance. Total DNA was isolated with Tri-Reagent as recommended by the manufacturer (Sigma-Aldrich) and plasmid DNA was isolated according to the alkaline lysis method (Sambrook et al., 1989). DNA was visualized after separation on 0.8% (w/v) TBE agarose gel by staining with fast blast DNA stain (Bio-Rad). DNA content and purity were measured by the method of Sambrook et al. (1989). Template DNA for PCR was obtained using the method of Whyte et al. (1996). For PCR amplification, 2 μ l of DNA extract was added to a final volume of 25 μ l reaction mixture, containing 5× GoTaq flexi buffer, MgCl₂, dNTP mix, specific primers (Table 1), and GoTaq DNA polymerase (Promega). PCR was performed with a C1000 thermal cycler (Bio-Rad). The PCR program consisted in initial denaturation for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 40 to 60°C for 30 s and an extension step at 72°C for 1 min, and a fi-

Table 1. PCR primers used in this study.

Primers	Sequence (5'–3')	PCR fragment size (bp)	Specificity	References
ALK-1f ALK-1r	5'-CATAATAAAGGGCATCACCGT-3' 5'-GATTTCATTCTCGAAACTCCAAAC-3'	185	Alkane hydroxylase Group I	Kohno et al., 2002
ALK-2f ALK-2r	5'-GAGACAAATCGTCTAAAACGTAA-3' 5'-TTGTTATTATTCCAATATGCTC-3'	271	Alkane hydroxylase Group II	Kohno et al., 2002
ALK-3f ALK-3r	5'-TCGAGCACATCCGCGGCCACCA-3' 5'-CCGTAGTGCTCGACGTAGTT-3'	330	Alkane hydroxylase Group III	Kohno et al., 2002
todC1-f todC1-r	5'-CGGGTGGGCTTACGACACCGCCGGAATCT-3' 5'-TCGAGCCGCGCTCCACGCTACCCAGACGTT-3'	560	Toluene dioxygenase	Whyte et al., 1996
TOL-f TOL-r	5'-TGAGGCTGAACTTTACGTAGA-3' 5'-CTCACCTGGAGTTGCGTAC-3'	475	Toluene/xylene monooxygenase	Baldwin et al., 2003
ISPGRLE1B ISPGRRI1B	5'-AAAGATCTGTACGGCG-3' 5'-TAAGCCCGGTAGAAACCACG-5'	900	PAH dioxygenase	Meyer et al., 1999
23CAT-f 23CAT-r	5'-CGACCTGATCTCCATGACCGA-3' 5'-TCAGGTCAGCACGGTCA-3'	238	Catechol 2,3-dioxygenase	Mesarch et al., 2000
A24f2 A577r2	5'-CCSRTITTYGCTGGGT-3' 5'-SAICCARAIRCAGCATSGC-3'	550	HAE1 transporter gene	Meguro et al., 2005
acrA-f acrB-r	5'-CGGTCATAACTTTCCAGACAGAGA-3' 5'-AAAACCTACTGACCTGGACTTGCC-3'	537–560 471–494	<i>acrAB</i> transporter gene	Tsukagoshi and Aono, 2000

Legend: f = forward PCR primer; r = reverse PCR primer; HAE1 = hydrophobe/amphiphile efflux 1.

nal extension at 72°C for 5 min. After separation on 1.5% (w/v) TBE agarose gel and staining with fast blast DNA stain the amplified fragments were analyzed.

Reagents. *n*-Hexane (96% pure), *n*-hexadecane (99% pure), cyclohexane (99.7% pure), benzene (99% pure), toluene (99% pure), styrene (99% pure), *o*-xylene (99% pure), *p*-xylene (99% pure), and propylbenzene (98% pure) were obtained from Merck; *n*-heptane (99% pure), *n*-decane (98% pure), *n*-pentadecane (98% pure), *m*-xylene (98% pure), ethylbenzene (98% pure), naphthalene (99% pure), 2-methylnaphthalene (97% pure), and fluorene (90% pure) were obtained from Sigma-Aldrich. Ampicillin, kanamycin, and rhodamine 6G were obtained from Sigma-Aldrich. Other reagents used were procured from Merck, Sigma-Aldrich, Difco Laboratories, Promega, bioMérieux or Bio-Rad Laboratories. The PCR primers were purchased from Biosearch Technologies and Integrated DNA Technologies.

Results and Discussion

In their natural habitats, living organisms are exposed to a wide range of natural and human-made toxic compounds, and survival in sites that have become hostile involves an extensive series of protective mechanisms. Although specific mechanisms of bacterial resistance to antimicrobials have been characterized and bypassed in certain cases, the more general mechanisms of altered cellular permeability to antimicrobial agents are much less understood on a physical and molecular basis. Addressing cellular per-

meability barriers by using methods for enhancing the uptake of antimicrobial agents could therefore represent an important means both in decreasing antibiotic usage levels and in preventing resistance, as alterations in permeability would be expected to involve a number of genes and require modification of the structurally complex cell membrane and cell wall structures of microorganisms (Brehm-Stecher and Johnson, 2003).

Isolation and characterization of bacterial strains

Bacteria with specific metabolic capabilities, such as hydrocarbon degradation, can be enumerated based on their ability to grow on selective media, using a 96-microwell plate MPN procedure (Haines et al., 1996). The number of total viable hydrocarbon-degrading bacteria per g of Poeni oily sludge was between 10^5 and 10^7 (data not shown). The isolation of bacterial strains from Poeni oily sludge was carried out on minimal medium, using the enriched cultures method. The use of minimal medium with 1% (v/v) Poeni crude oil as the single carbon source allowed the selective isolation of six Gram-positive (Po₁–Po₅, Po₇) and eight Gram-negative (Po₆, Po₈–Po₁₄) hydrocarbon-degrading bacteria. The taxonomic affiliation of isolated bacteria was determined based on the phenotypic characteristics, the G+C content of the bacterial chromosome (data not shown) and their 16S rDNA sequences (Table 2). The taxonomic affiliation of Po₈ strain was determined based on the phenotypic characteristics and the G+C content of the bacterial chromosome. Based on the API profile (3457754) and on

Table 2. Taxonomic affiliation of isolated bacterial strains based on the 16S rDNA sequence.

Strain name	Length of sequenced part (bases)	Closest bacterial strain name (accession number)	Percentage of nucleotide identity (%)
<i>Bacillus</i> sp. Po ₁	1,369	<i>Bacillus</i> sp. PaH3.34a2 (GQ406796.1)	99
<i>Bacillus</i> sp. Po ₂	1,365	<i>Bacillus thuringiensis</i> strain TCCC11336 (FJ393313.1)	95
<i>Lysinibacillus</i> sp. Po ₃	1,364	<i>Lysinibacillus fusiformis</i> strain BJ-25 (GQ280035.1)	99
<i>Rhodococcus</i> sp. Po ₄	1,331	<i>Rhodococcus</i> sp. DSP-CA (AB547168.1)	99
<i>Rhodococcus</i> sp. Po ₅	1,327	<i>Rhodococcus globerulus</i> strain AZ1-15 (GU332596.1)	99
<i>Shewanella</i> sp. Po ₆	1,354	<i>Shewanella</i> sp. OTUC2 (FJ210843.1)	99
<i>Lysinibacillus</i> sp. Po ₇	1,353	<i>Lysinibacillus fusiformis</i> strain IMAUB1017 (FJ641029.1)	99
<i>Aeromonas</i> sp. Po ₈	ND ^a	ND	ND
<i>Pseudomonas</i> sp. Po ₉	610	<i>Pseudomonas putida</i> strain KT 821 (GU459173.1)	99
<i>Pseudomonas</i> sp. Po ₁₀	1,344	<i>Pseudomonas aeruginosa</i> strain F5-2 (GQ921945.1)	99
<i>Pseudomonas</i> sp. Po ₁₁	1,347	<i>Pseudomonas putida</i> strain CDd-9 (GU248219.1)	99
<i>Klebsiella</i> sp. Po ₁₂	1,353	<i>Klebsiella</i> sp. XW721 (EU545402.1)	99
<i>Shewanella</i> sp. Po ₁₃	1,352	<i>Shewanella algae</i> strain MAS2737 (GQ372875.1)	99
<i>Pseudomonas</i> sp. Po ₁₄	1,338	<i>Pseudomonas aeruginosa</i> clone X3 (AY631240.1)	99

^aND = not determined.

the 61.5 mol% G+C content of the DNA, the Po₈ strain was identified as *Aeromonas* sp.

Isolated bacteria could be included in phylum *Firmicutes* (*Bacillus* sp. Po₁, Po₂, *Lysinibacillus* sp. Po₃, Po₇), phylum *Actinobacteria* (*Rhodococcus* sp. Po₄, Po₅) and phylum *Proteobacteria* (Gamma-subclass of *Proteobacteria*—*Shewanella* sp. Po₆, Po₁₃, *Pseudomonas* sp. Po₉, Po₁₀, Po₁₁, Po₁₄, *Klebsiella* sp. Po₁₂ and Delta-subclass of *Proteobacteria*—*Aeromonas* sp. Po₈). Although many bacteria are capable of degrading petroleum hydrocarbons, only few of them are considered of importance for petroleum biodegradation in natural environments. The repeated isolation of different bacteria belonging to *Proteobacteria*, *Actinobacteria* and *Firmicutes* from sites contaminated with poorly available and recalcitrant pollutants, suggests that

members of these taxa may be physiologically favored in polluted environments compared to other bacteria (Hamamura et al., 2006; Kalscheuer et al., 2007; Segura et al., 2008; Sokolovská et al., 2003; Zahir et al., 2006).

Resistance of isolated bacteria to hydrophilic antibiotics and toxic compounds

We tested the resistance of isolated bacteria to hydrophilic antibiotics like ampicillin and kanamycin with log P_{OW} (the logarithm of the partition coefficient of the antibiotic in octanol–water mixture) -1.85 and -7.77 , respectively (Aono et al., 1998; Asako et al., 1997; Zimnitsky et al., 2006). The tested antibiotics differ in their mode of action, as well as in their chemical structures and physical properties. The resistance of iso-

Table 3. The resistance of Gram-positive and Gram-negative bacteria to hydrophilic antibiotics and toxic compounds.

Bacterial strain		MIC ₉₀ (μg ml ⁻¹) of:					
		Ampicillin	Kanamycin	Rhodamine 6G	Crystal violet	Malachite green	Sodium dodecyl sulfate
<i>Bacillus</i> sp. Po ₁	C ^{BS}	500	50	100	100	250	40
	Amp ₂₅	500	50	100	100	250	40
<i>Bacillus</i> sp. Po ₂	C ^{BS}	800	50	100	200	25	75
	Amp ₂₅	800	50	100	200	25	75
<i>Lysinibacillus</i> sp. Po ₃	C ^{BS}	250	75	100	180	25	50
	Km ₂₅	250	75	100	180	25	50
<i>Rhodococcus</i> sp. Po ₄	C ^{BS}	250	100	50	100	50	50
	Km ₂₅	250	100	50	100	50	50
<i>Rhodococcus</i> sp. Po ₅	C ^{BS}	250	100	50	100	50	50
	Km ₂₅	250	100	50	100	50	50
<i>Shewanella</i> sp. Po ₆	C ^{BS}	1,000	25	1,000	750	25	1,000
	Amp ₂₅	1,000	25	1,000	750	25	1,000
<i>Lysinibacillus</i> sp. Po ₇	C ^{BS}	1,000	45	1,000	500	500	1,000
	Amp ₂₅	1,000	45	1,000	500	500	1,000
<i>Aeromonas</i> sp. Po ₈	C ^{BS}	1,000	35	1,000	1,000	300	1,000
	Amp ₂₅	1,000	35	1,000	1,000	300	1,000
<i>Pseudomonas</i> sp. Po ₉	C ^{BS}	1,000	45	1,000	1,000	1,000	1,000
	Amp ₂₅	1,000	45	1,000	1,000	1,000	1,000
<i>Pseudomonas</i> sp. Po ₁₀	C ^{BS}	1,000	550	1,000	1,000	1,000	1,000
	Km ₂₅	1,000	550	1,000	1,000	1,000	1,000
<i>Pseudomonas</i> sp. Po ₁₁	C ^{BS}	750	100	1,000	500	1,000	1,000
	Amp ₂₅	750	100	1,000	500	1,000	1,000
<i>Klebsiella</i> sp. Po ₁₂	C ^{BS}	1,000	250	1,000	1,000	1,000	1,000
	Amp ₂₅	1,000	250	1,000	1,000	1,000	1,000
<i>Shewanella</i> sp. Po ₁₃	C ^{BS}	1,000	25	1,000	750	300	1,000
	Amp ₂₅	1,000	25	1,000	750	300	1,000
<i>Pseudomonas</i> sp. Po ₁₄	C ^{BS}	1,000	500	1,000	1,000	1,000	1,000
	Km ₂₅	1,000	500	1,000	1,000	1,000	1,000

C^{BS} = bacterial strains cultivated in liquid LB-Mg medium; Amp₂₅, Km₂₅ = bacterial strains cultivated in liquid LB-Mg medium supplemented with 25 μg ml⁻¹ ampicillin and kanamycin, respectively; MIC₉₀ = Minimum Inhibitory Concentration required to inhibit the growth of 90% of the bacteria.

lated bacteria to toxic compounds like rhodamine 6G, crystal violet, malachite green, and sodium dodecyl sulfate was also tested in the present study. The resistance of isolated bacteria to hydrophilic antibiotics and toxic compounds differs from one strain to another (Table 3). The MIC₉₀ for Gram-positive bacteria (except *Lysinibacillus* sp. Po₇) was between 25 and 800 µg ml⁻¹, whereas the MIC₉₀ for Gram-negative bacteria was between 25 and 1,000 µg ml⁻¹. *Lysinibacillus* sp. Po₇ was found to be more resistant to hydrophilic antibiotics and toxic compounds (MIC₉₀ = 45–1,000 µg ml⁻¹) than the other Gram-positive bacteria (MIC₉₀ = 25–800 µg ml⁻¹). No modifications were observed in the MIC₉₀ values for Gram-positive and Gram-negative bacteria grown in liquid LB-Mg medium supplemented with 25 µg ml⁻¹ ampicillin or kanamycin, compared with the control. Many bacteria resistant to antibiotics and toxic compounds have been isolated from polluted and unpolluted sites. However, bacterial strains isolated from polluted environments are more resistant to toxic compounds (MIC values are significantly higher), compared with bacteria isolated from unpolluted sites (Li et al., 2010).

Previously, Li et al. (2009) investigated the bacterial characteristics of a penicillin production wastewater treatment plant and the receiving river and demonstrated that high resistance prevalence and levels could be induced by long-term penicillin exposure. The resistance ratios and levels significantly differed among strains belonging to different species (Li et al., 2009).

Gram-positive and Gram-negative bacteria were more susceptible to kanamycin (MIC₉₀ = 25–550 µg ml⁻¹), if compared with ampicillin (MIC₉₀ = 250–1,000 µg ml⁻¹). Belonging to the penicillin group of β-lactam antibiotics, ampicillin is able to penetrate membranes of Gram-positive and some Gram-negative bacteria. Ampicillin acts as a competitive inhibitor of the enzyme transpeptidase used by bacteria to make their cell walls. It inhibits the third and final stage of the bacterial cell wall synthesis process, ultimately leading to cell lysis. On the other hand, kanamycin, as a member of the aminoglycoside group of antibiotics, works by affecting the 30S ribosomal subunit and causing a frameshift mutation or by preventing the translation of RNA. Therefore, the needed protein cannot be synthesized. Depending on the site and severity of the frame shift, either a completely different protein is synthesized, or a protein similar to the needed one is synthe-

sized but is incorrectly folded. The bacterium is destroyed because it cannot produce correctly any of its proteins (Depardieu et al., 2007).

Accumulation of rhodamine 6G (5–1,000 µg ml⁻¹) in Gram-positive and Gram-negative bacterial cells was determined by observing the fluorescence of rhodamine 6G under UV light. Rhodamine 6G is the P-glycoprotein (phosphoglycoprotein) substrate, which mediates the energy-dependent efflux of certain toxic compounds, from the bacterial cells (Nishino and Yamaguchi, 2004). The P-glycoprotein, a well characterized ABC-transporter, was demonstrated to bind a large number of lipophilic compounds (i.e., antibiotics, dyes, polyaromatic hydrocarbons, polychlorinated biphenyls, heavy metals, pesticides, and other toxic compounds) with no apparent structural or functional similarities (Borges-Walmsley et al., 2003; Sikkema et al., 1995). Both Gram-positive and Gram-negative bacteria tolerate the presence of rhodamine 6G in the culture medium and they also accumulate this toxic compound inside the cells (Table 3, Fig. 1). Gram-negative bacteria were more tolerant (MIC₉₀ = 1,000 µg ml⁻¹) to rhodamine 6G than Gram-positive bacteria (except *Lysinibacillus* sp. Po₇) (MIC₉₀ = 50–100 µg ml⁻¹). The MIC₉₀ value for *Lysinibacillus* sp. Po₇ was higher (1,000 µg ml⁻¹) than the MIC₉₀ of other Gram-positive bacteria (MIC₉₀ = 50–100 µg ml⁻¹). The rhodamine 6G accumulation in Gram-negative bacteria was higher (75–100%) than its accumulation in Gram-positive bacteria (0–50%).

The antimicrobial effect of all tested compounds (i.e., ampicillin, kanamycin, rhodamine 6G, crystal violet, malachite green, and sodium dodecyl sulfate) was more pronounced in the case of Gram-positive bacteria (except *Lysinibacillus* sp. Po₇), probably due to the lack of additional permeability barriers, and particularly the outer membrane present in Gram-negative bacteria. Nevertheless, the outer membrane itself does not provide resistance to antimicrobial agents as it only decreases outer membrane permeability, impairing their penetration of the cell (Poole, 2001). The resistance of Gram-negative bacteria to antimicrobial agents is due to other resistance mechanisms, such as efflux systems, but these mechanisms have enhanced effectiveness in the presence of the outer membrane. The synergy between the efflux pumps and the outer membrane probably explains the variable effectiveness of related multidrug efflux systems in providing resistance in organisms with differences

Fig. 1. Rhodamine 6G accumulation in Gram-positive and Gram-negative bacterial cells in the presence of ampicillin or kanamycin.

After incubation at 28°C for 24 h, rhodamine 6G accumulation in bacterial cells was observed under visible white light (panel a, b, e, f, i, j) and UV light (panel c, d, g, h, k, l); *Bacillus* sp. Po₁ (spots 1, 2), *Bacillus* sp. Po₂ (spots 3, 4), *Lysinibacillus* sp. Po₃ (spots 5, 6), *Rhodococcus* sp. Po₄ (spots 7, 8), *Rhodococcus* sp. Po₅ (spots 9, 10), *Shewanella* sp. Po₆ (spots 11, 12), *Lysinibacillus* sp. Po₇ (spots 13, 14), *Aeromonas* sp. Po₈ (spots 15, 16), *Pseudomonas* sp. Po₉ (spots 17, 18), *Pseudomonas* sp. Po₁₀ (spots 19, 20), *Pseudomonas* sp. Po₁₁ (spots 21, 22), *Klebsiella* sp. Po₁₂ (spots 23, 24), *Shewanella* sp. Po₁₃ (spots 25, 26), *Pseudomonas* sp. Po₁₄ (spots 27, 28); bacterial strains cultivated in liquid LB-Mg medium (spots 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27); bacterial strains cultivated in liquid LB-Mg medium supplemented with 25 µg ml⁻¹ ampicillin (spots 2, 4, 12, 14, 16, 18, 22, 24, 26) and 25 µg ml⁻¹ kanamycin (spots 6, 8, 10, 20, 28).

in intrinsic outer membrane permeability properties (Poole, 2001, 2005).

Effects of hydrophilic antibiotics on bacteria tolerance to saturated, monoaromatic, and polyaromatic hydrocarbons

The key factor for hydrocarbon degradation is represented by the tolerance that some microorganisms exhibit toward these compounds. It is generally assumed that hydrocarbons exhibit extreme toxicity toward living microorganisms, because of their accumulation in the hydrophobic biological membranes. The hydrophobicity of hydrocarbons can be expressed in terms of log P_{OW} , which represents the ability of a compound to partition over an octanol–water two-phase system (Sikkema et al., 1994). It has been established that log P_{OW} is correlated with the toxicity of a specific hydrocarbon and that log P_{OW} values, especially between two and four, are highly toxic for microorganisms (Osborne et al., 1990), and within this range, those with lower log P_{OW} values are considered more toxic than those with higher log P_{OW} values (Inoue and Horikoshi, 1991). Whether a hydrocarbon is toxic to a bacterial cell depends upon its concentration in the membrane, which relates to its water solubility and its ability to partition from the water phase to the mem-

brane (Sikkema et al., 1994). Hydrocarbons with log P_{OW} below two are generally too hydrophilic to partition into the membranes, and hydrocarbons with log P_{OW} above four are too hydrophobic to have high water solubility (Heipieper et al., 2007; Zahir et al., 2006).

The isolated bacteria were able to tolerate saturated, monoaromatic, and polyaromatic hydrocarbons (Table 4). The tolerance of Gram-positive and Gram-negative bacteria to saturated (*n*-hexane, *n*-heptane, *n*-decane, *n*-pentadecane, *n*-hexadecane, and cyclohexane), monoaromatic (benzene, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene), and polyaromatic (naphthalene, 2-methylnaphthalene, and fluorene) hydrocarbons differs from one strain to another and even for the same bacterial strain according to the nature of the hydrophobic substrate. Hydrocarbon toxicity depends not only on its physico-chemical properties but also on the specific response of the cells, and that is why the cellular response is not the same in all strains (Segura et al., 2008). The growth of Gram-positive bacteria on LB-Mg agar medium supplied with hydrocarbons in the vapor phase was 10–100% for saturated hydrocarbons and polyaromatic hydrocarbons and only 10–50% for monoaromatic hydrocarbons. The growth of Gram-negative bacteria on LB-Mg agar medium supplied with hydro-

Table 4. The effects of ampicillin or kanamycin on Gram-positive and Gram-negative bacteria tolerance to saturated, monoaromatic, and polyaromatic hydrocarbons.

Bacterial strain		Growth of the strains ^a on solid LB-Mg medium with hydrocarbons (log P_{OW}):																	
		C^H	Saturated							Monoaromatic							Polyaromatic		
			Hex	Hept	Dec	PDec	HDec	CHex	Bnz	Tol	Sty	<i>o</i> -Xyl	<i>m</i> -Xyl	<i>p</i> -Xyl	EBnz	PBnz	Nah	MNah	Flu
			-	(3.86)	(4.39)	(5.98)	(8.62)	(9.15)	(3.35)	(2.14)	(2.64)	(2.86)	(3.09)	(3.14)	(3.14)	(3.17)	(3.69)	(3.31)	(3.81)
<i>Bacillus</i> sp. Po ₁	C^{BS}	100	75	10	100	100	100	75	50	10	25	10	50	50	25	50	75	50	100
	Amp ₂₅	100	75	10	100	100	100	75	50	10	50	10	50	50	50	50	75	50	100
<i>Bacillus</i> sp. Po ₂	C^{BS}	100	100	100	100	100	100	50	25	10	50	10	50	50	50	50	100	50	100
	Amp ₂₅	100	100	100	100	100	100	50	25	10	50	10	50	50	50	50	100	50	100
<i>Lysinibacillus</i> sp. Po ₃	C^{BS}	100	100	100	100	100	100	10	10	10	10	10	25	50	25	50	100	50	100
	Km ₂₅	100	100	100	100	100	100	10	10	10	10	10	25	50	25	50	100	50	100
<i>Rhodococcus</i> sp. Po ₄	C^{BS}	100	25	10	10	100	100	25	10	10	10	10	25	25	25	25	25	25	75
	Km ₂₅	100	25	10	10	100	100	25	10	10	10	10	25	25	25	25	25	25	75
<i>Rhodococcus</i> sp. Po ₅	C^{BS}	100	25	10	10	100	100	25	10	10	10	10	25	25	25	50	25	25	75
	Km ₂₅	100	25	10	10	100	100	25	10	10	10	10	25	25	25	50	25	25	75
<i>Shewanella</i> sp. Po ₆	C^{BS}	100	10	10	100	100	100	10	10	10	10	10	25	25	25	50	10	10	100
	Amp ₂₅	100	10	10	100	100	100	10	10	10	10	10	25	25	25	50	10	10	100
<i>Lysinibacillus</i> sp. Po ₇	C^{BS}	100	10	100	100	100	100	10	10	10	10	10	10	25	10	10	10	25	100
	Amp ₂₅	100	10	100	100	100	100	10	10	10	10	10	10	25	10	10	10	25	100
<i>Aeromonas</i> sp. Po ₈	C^{BS}	100	75	100	100	100	100	10	10	10	10	25	25	25	10	10	10	50	100
	Amp ₂₅	100	75	100	100	100	100	10	10	10	10	25	25	25	10	10	10	50	100
<i>Pseudomonas</i> sp. Po ₉	C^{BS}	100	100	100	100	100	100	100	10	10	10	100	100	50	100	100	100	100	100
	Amp ₂₅	100	100	100	100	100	100	100	10	10	10	100	100	50	100	100	100	100	100
<i>Pseudomonas</i> sp. Po ₁₀	C^{BS}	100	10	50	100	100	100	100	10	10	10	100	100	25	100	100	100	100	100
	Km ₂₅	100	10	50	100	100	100	100	10	10	10	100	100	25	100	100	100	100	100
<i>Pseudomonas</i> sp. Po ₁₁	C^{BS}	100	100	100	100	100	100	100	10	10	10	100	100	50	100	100	100	100	100
	Amp ₂₅	100	100	100	100	100	100	100	10	10	10	100	100	50	100	100	100	100	100
<i>Klebsiella</i> sp. Po ₁₂	C^{BS}	100	100	100	100	100	100	100	10	10	10	100	100	50	100	100	100	100	100
	Amp ₂₅	100	100	100	100	100	100	100	10	10	10	100	100	50	100	100	100	100	100
<i>Shewanella</i> sp. Po ₁₃	C^{BS}	100	100	100	100	100	100	25	10	10	10	10	10	25	10	10	10	50	100
	Amp ₂₅	100	100	100	100	100	100	25	10	10	10	10	10	25	10	10	10	50	100
<i>Pseudomonas</i> sp. Po ₁₄	C^{BS}	100	50	75	100	100	100	100	10	10	10	100	100	25	100	100	100	100	100
	Km ₂₅	100	50	75	100	100	100	100	10	10	10	100	100	25	100	100	100	100	100

C^{BS} = bacterial strains cultivated in liquid LB-Mg medium; Amp₂₅, Km₂₅ = bacterial strains cultivated in liquid LB-Mg medium supplemented with 25 $\mu\text{g ml}^{-1}$ ampicillin, kanamycin respectively; ^a = the growth on solid LB-Mg medium was estimated by determining the formation of resistant bacterial colonies and the hydrocarbon resistance is represented by the frequency of colony formation, with that observed in the absence of any hydrocarbon taken as 100%; log P_{OW} = logarithm of the partition coefficient of the hydrocarbons in octanol-water mixture; C^H = bacterial cells were spotted on solid LB-Mg medium (control) and on the same medium with hydrocarbons (Hex = *n*-hexane, Hept = *n*-heptane, Dec = *n*-decane, PDec = *n*-pentadecane, HDec = *n*-hexadecane, CHex = cyclohexane, Bnz = benzene, Tol = toluene, Sty = styrene, *o*-Xyl = *o*-xylene, *m*-Xyl = *m*-xylene, *p*-Xyl = *p*-xylene, EBnz = ethylbenzene, PBnz = propylbenzene, Nah = naphthalene, MNah = 2-methylnaphthalene, Flu = fluorene) supplied in the vapor phase.

carbons in the vapor phase or incorporated into solid medium was 10–100% for saturated, monoaromatic, and polyaromatic hydrocarbons. It is well known that bacteria sometimes exhibit cross-protection when they are exposed to different stresses (Isken et al., 1997; Segura et al., 2008). However, the presence of 25 $\mu\text{g ml}^{-1}$ ampicillin or kanamycin in liquid LB-Mg medium did not enhance effects on isolated Gram-positive and Gram-negative bacteria tolerance to satu-

rated, monoaromatic, or polyaromatic hydrocarbons. We observed, like in the previous assay, that Gram-negative bacteria were less sensitive to toxic compounds than Gram-positive bacteria (except *Lysinibacillus* sp. Po₇).

We previously reported (Lăzăroaie, 2010) similar results for six Gram-positive and Gram-negative strains. Isolation of these bacterial strains was done from a different Poeni oily sludge than the one used in the pres-

ent study, using minimal medium supplemented with saturated and aromatic hydrocarbon mixtures as the single carbon source. The Gram-negative bacteria (*Chryseomonas*, *Pseudomonas*, *Burkholderia* genera) were less sensitive to toxic compounds (i.e., mixture of saturated and aromatic hydrocarbons, ampicillin, kanamycin, sodium dodecyl sulfate, and rhodamine 6G), compared with Gram-positive bacteria (*Mycobacterium*, *Oerskovia*, *Corynebacterium* genera) (Lăzăroaie, 2010). Because of the highly impermeable outer membrane of Gram-negative bacteria, it is generally accepted that this type of bacteria is more tolerant to hydrocarbons than Gram-positive bacteria (Inoue and Horikoshi, 1991; Isken et al., 1997; Vermuë et al., 1993). Modification of the outer membrane of Gram-negative bacteria is often related to its LPS composition. The outer membrane is a highly porous shield that allows small hydrophilic solutes to pass via its pores. However, it has a surprisingly high transfer resistance to hydrophobic compounds. This transfer resistance has been shown to be a result of the highly hydrophilic LPS. It can be envisaged that some strains of Gram-negative bacteria are able to further increase the transfer resistance to lipophilic compounds via either an induction mechanism or a mutagenic event (Nikaido, 1996, 1998, 2003). Nevertheless, several Gram-positive bacteria seem to be more resistant to hydrocarbons, compared with Gram-negative bacteria (Segura et al., 2008; Zahir et al., 2006).

Cellular and molecular modifications induced by hydrophilic antibiotics on isolated bacteria

There are studies showing that antibiotics, in addition to their effects on primary targets such as peptidoglycan or protein synthesis or DNA supercoiling, have multiple secondary effects on the bacterial cell, especially on its membrane or envelope (Suerbaum et al., 1987). In order to reveal possible secondary effects of hydrophilic antibiotics like ampicillin or kanamycin on isolated bacteria, cell viability, β -galactosidase activity, lipid profile, protein profile and DNA were examined. For most of the isolated bacteria (*Bacillus* sp. Po₁, Po₂, *Lysinibacillus* sp. Po₇, *Shewanella* sp. Po₆, Po₁₃, *Aeromonas* sp. Po₈, *Pseudomonas* sp. Po₉, Po₁₁, *Klebsiella* sp. Po₁₂) we investigated the modifications induced by 25 $\mu\text{g ml}^{-1}$ ampicillin, while for five bacteria (*Lysinibacillus* sp. Po₃, *Rhodococcus* sp. Po₄, Po₅, *Pseudomonas* sp. Po₁₀, Po₁₄) we investigated the modifications induced by 25 $\mu\text{g ml}^{-1}$ of both ampicillin and kanamycin.

As there were no significant differences between the modifications induced on cell viability, β -galactosidase activity, lipids, proteins and DNA extracted from bacterial cells incubated in the presence of ampicillin and kanamycin, the only results presented in this paper, for these five bacterial strains, are the results obtained for cells incubated in the presence of kanamycin.

Modifications to cells viability induced by antibiotics

The maintenance or loss of viability of Gram-positive and Gram-negative bacterial cells grown in the absence (control) or in the presence of 25 $\mu\text{g ml}^{-1}$ ampicillin or kanamycin, estimated by determining the MPN of bacterial cells on solid medium (with values between 10^4 and 10^{11} CFU ml^{-1}), were correlated with growth on liquid medium (OD₆₆₀ with values between 0.500 and 1.869) (data not shown). The survival rate in the presence of 25 $\mu\text{g ml}^{-1}$ ampicillin or kanamycin was lower (10^4 – 10^8 CFU ml^{-1}) for Gram-positive bacteria, compared with the survival rate of Gram-negative bacteria (10^6 – 10^{10} CFU ml^{-1}) (data not shown). Gram-negative bacteria were less sensitive to antibiotics than Gram-positive bacteria (except *Lysinibacillus* sp. Po₇).

Effects of antibiotics to β -galactosidase activity

After bacterial cells permeabilization with chloroform and sodium dodecyl sulfate, *o*-nitrophenyl- β -D-galactoside diffused into the cells and it was hydrolyzed by the β -galactosidase enzyme entrapped within the cell. We observed that Gram-negative bacteria were β -galactosidase-positive, while Gram-positive bacteria were β -galactosidase-negative, except *Bacillus* sp. Po₁ which was β -galactosidase-positive (data not shown). The level of β -galactosidase activity was measured in the late exponential phase of the growth curve, in the presence and in the absence of 25 $\mu\text{g ml}^{-1}$ ampicillin or kanamycin. β -galactosidase activity measurements revealed that the *lacZ* gene was induced in bacterial cells grown in the presence of 25 $\mu\text{g ml}^{-1}$ ampicillin or kanamycin (data not shown). Antibiotics induced β -galactosidase activity in Gram-negative bacterial cells to a level between 1.2 and 4.4 times greater than the basal level, while in Gram-positive *Bacillus* sp. Po₁ cells the induction was 2.0 times greater than the basal level. Similar results were previously obtained by Kieboom et al. (1998) for *Pseudomonas putida* S12. The level of β -galactosidase activity was measured in the late exponential phase of cultures

exposed to different antibiotics resulting in approximately 50% decrease in the growth rate. Growth of *Pseudomonas putida* S12 (pKRZ-srp) in the presence of 128 $\mu\text{g ml}^{-1}$ chloramphenicol, 128 $\mu\text{g ml}^{-1}$ ampicillin, and 4 $\mu\text{g ml}^{-1}$ tetracycline resulted in a twofold increase in induction over basal levels. No increase in induction over basal levels was observed in the presence of the other antibiotics tested (i.e., 256 $\mu\text{g ml}^{-1}$ penicillin G, 256 $\mu\text{g ml}^{-1}$ novobiocin, and 4 $\mu\text{g ml}^{-1}$ rifampin) (Kieboom et al., 1998).

Modifications to lipids induced by antibiotics

Cell membranes constitute the first biological structure encountered by antibiotics. Interactions of antibiotics with lipid components of the membrane may lead to toxic events (http://www.facm.ucl.ac.be/drug_membranes.htm).

The thin-layer chromatography (TLC) studies revealed the existence of some differences (motilities and phospholipid headgroup compositions) between phospholipids extracted from Gram-positive and Gram-negative bacterial cells incubated without antibiotic (control) and those extracted from cells incubated in the presence of 25 $\mu\text{g ml}^{-1}$ ampicillin or kanamycin (Fig. 2a). The phospholipids identified, based on their motilities (R_f) in Gram-positive and Gram-negative bacterial cells were lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and cardiolipin (CL). The phospholipid found, based on their motilities (R_f), in Gram-positive *Bacillus* sp. Po₁, Po₂, *Lysinibacillus* sp. Po₃ cells (control) was CL (R_f = 0.96–0.98). No significant modifications were observed in the lipid profile of these Gram-positive cells grown in the presence of 25 $\mu\text{g ml}^{-1}$ ampicillin or kanamycin, compared with the control. The phospholipids found in Gram-positive (*Rhodococcus* sp. Po₄, Po₅, *Lysinibacillus* sp. Po₇) and Gram-negative (*Shewanella* sp. Po₆, Po₁₃, *Aeromonas* sp. Po₈, *Pseudomonas* sp. Po₉, Po₁₀, Po₁₁, Po₁₄, *Klebsiella* sp. Po₁₂) cells (control) were LPC (R_f = 0.09–0.11), PC (R_f = 0.17–0.21), PS (R_f = 0.26), PE (R_f = 0.34–0.44), PG (R_f = 0.61), PI (R_f = 0.56–0.75) and CL (R_f = 0.92–0.97). The phospholipids found in these Gram-positive and Gram-negative bacterial cells incubated in the presence of 25 $\mu\text{g ml}^{-1}$ ampicillin or kanamycin were LPC (R_f = 0.09–0.10), PC (R_f = 0.15–0.22), PS (R_f = 0.24–0.28), PE (R_f = 0.36–0.46), PG (R_f = 0.57), and PI (R_f = 0.59–0.76) and CL (R_f =

0.94–0.98).

The data available in the literature with regards to the changes in the lipid composition of the cell envelope of bacteria during the development of their resistance to antibiotics are scarce and often contradictory (Kuyukina et al., 2000). Monolayer studies with pure lipid extracts from different strains showed that antibiotics interacted more efficiently with lipids derived from some bacterial strains than with others, reflecting qualitative differences in antibiotic sensitivity (Verheul et al., 1997). In addition to membrane abnormalities caused by specific inhibitors of lipid synthesis, structural changes in cell wall lipids occur in response to various stress conditions. Temperature shifts, starvation, low pH, and hydrocarbons can modify the ratios of saturated to unsaturated fatty acids or induce *cis-trans* isomerization and cyclopropanation. Alterations of the phospholipid headgroups had an additional effect on the physico-chemical properties of the membrane. Generally, an enhanced membrane phospholipid biosynthesis in cells upon different stress conditions could be observed. These changes are interpreted as a way for bacteria to maintain membrane fluidity and impermeability (Ramos et al., 2002; Sikkeima et al., 1995; Sokolovská et al., 2003).

Modifications to proteins induced by antibiotics

To investigate the modifications induced by ampicillin or kanamycin to the membrane (for Gram-positive and Gram-negative bacteria) and periplasmic (for Gram-negative bacteria) protein profile, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS-PAGE) was used. Many more types of variation in protein sequences can be distinguished on one-dimensional gels in the absence of denaturants such as urea used in two-dimensional electrophoresis (McLellan et al., 1983). The electrophoresis studies showed the existence of some differences between protein profiles extracted from Gram-positive and Gram-negative bacterial cells incubated without antibiotics (control) and those extracted from cells incubated in the presence of 25 $\mu\text{g ml}^{-1}$ ampicillin or kanamycin. The induced modifications were different from one strain to another (Fig. 2b). We observed the induction of the synthesis of some proteins in the protein profile of Gram-positive *Bacillus* sp. Po₂, *Lysinibacillus* sp. Po₃ cells grown in the presence of antibiotics, the repression of the synthesis of some proteins was observed in *Lysinibacillus* sp. Po₇ cells, and no

Fig. 2. The lipid profile (a), protein profile (b) and DNA (c) of Gram-positive and Gram-negative bacteria in the presence of ampicillin or kanamycin.

Bacillus sp. Po₁ (lanes 1, 2), *Bacillus* sp. Po₂ (lanes 3, 4), *Lysinibacillus* sp. Po₃ (lanes 5, 6), *Rhodococcus* sp. Po₄ (lanes 7, 8), *Rhodococcus* sp. Po₅ (lanes 9, 10), *Shewanella* sp. Po₆ (lanes 11, 12), *Lysinibacillus* sp. Po₇ (lanes 13, 14), *Aeromonas* sp. Po₈ (lanes 15, 16), *Pseudomonas* sp. Po₉ (lanes 17, 18), *Pseudomonas* sp. Po₁₀ (lanes 19, 20), *Pseudomonas* sp. Po₁₁ (lanes 21, 22), *Klebsiella* sp. Po₁₂ (lanes 23, 24), *Shewanella* sp. Po₁₃ (lanes 25, 26), *Pseudomonas* sp. Po₁₄ (lanes 27, 28); bacterial strains cultivated in liquid LB-Mg medium (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27); bacterial strains cultivated in liquid LB-Mg medium supplemented with 25 $\mu\text{g ml}^{-1}$ ampicillin (lanes 2, 4, 12, 14, 16, 18, 22, 24, 26) and 25 $\mu\text{g ml}^{-1}$ kanamycin (lanes 6, 8, 10, 20, 28). Panel a. Phospholipids standards, Sigma-Aldrich, Supelco (lane PLS); origin (O), solvent front (F), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), cardiolipin (CL). Panel b. Broad range protein molecular weight marker, Promega (lane M). Panel c. 1 kb DNA ladder, Promega (lane M₁), negative control DNA (lane 29), the positions of the expected HAE1 fragment are indicated with arrow.

modifications were observed in *Bacillus* sp. Po₁, *Rhodococcus* sp. Po₄, Po₅ cells, compared with the control. Similarly, in Gram-negative bacteria grown in the presence of antibiotics, other proteins than the one observed in Gram-positive bacteria were induced in some strains (*Shewanella* sp. Po₆, *Aeromonas* sp. Po₈, *Klebsiella* sp. Po₁₂, *Pseudomonas* sp. Po₁₄), while the repression of others was observed in *Pseudomonas* sp. Po₁₀, Po₁₁ and *Shewanella* sp. Po₁₃. No modifications were observed in the membrane or periplasmic protein profile of *Pseudomonas* sp. Po₉.

The induction and repression of a great number of proteins in response to various stress conditions has already been described (Isken et al., 1997; Poole, 2005; Ramos et al., 2002; Segura et al., 1999, 2005; Terán et al., 2003; Viveiros et al., 2007). The MexXY-OprM system of *Pseudomonas aeruginosa*, for example, is both induced by and exports several antibiotics, including gentamicin, erythromycin and tetracycline and, as such, efflux of these agents may be its intended function. Still, it is also possible that the action of these agents on their ribosome targets induces expression of the MexXY-OprM efflux system as a result of the accumulation of cellular products whose export is carried out by this efflux system (Poole, 2005). The MexCD-OprJ efflux system of the same organism is also inducible by a number of non-antibiotic substrates such as rhodamine 6G, acriflavine and ethidium bromide (Morita et al., 2001). In addition to the transcriptional regulation of genes coding for membrane proteins, the post-translational regulation of proteins involved in the permeability of Gram-negative bacteria also plays a major role in the physiological adaptation to antibiotic exposure (Viveiros et al., 2007). Adaptation to a certain hydrocarbon resulted in concomitant tolerance towards other hydrocarbons and increased resistance to antibiotics and heavy metals (Isken et al., 1997).

Screening of isolated bacteria for the presence of catabolic genes and transporter genes

We observed, by analyzing the total and plasmid DNA, the existence of some plasmids in the Gram-positive (*Lysinibacillus* sp. Po₃, Po₇, *Rhodococcus* sp. Po₄) and Gram-negative (*Shewanella* sp. Po₆, Po₁₃, *Aeromonas* sp. Po₈, *Pseudomonas* sp. Po₉, Po₁₀, Po₁₁, Po₁₄) bacteria incubated without antibiotics (control) and those incubated in the presence of 25 µg ml⁻¹ ampicillin or kanamycin (data not shown). Despite the

high tolerance of Gram-positive (*Bacillus* sp. Po₁, Po₂, *Rhodococcus* sp. Po₅) and Gram-negative (*Klebsiella* sp. Po₁₂) bacteria to saturated, monoaromatic, and polyaromatic hydrocarbons and antimicrobial compounds, these bacteria do not harbor plasmids (data not shown). Numerous plasmid- and chromosome encoded efflux mechanisms, both agent- or class-specific and multidrug have been described in a variety of organisms where they are increasingly appreciated as important determinants of antimicrobial resistance (Poole, 2005).

Microorganisms which biodegrade different hydrocarbons were readily isolated from petroleum-contaminated sites (Cerniglia, 1993; Márquez-Rocha et al., 2005; Whyte et al., 1996). Catabolic genes might be used in systematic studies to evaluate the biodegradation potential of isolates from contaminated sites (Márquez-Rocha et al., 2005). The ability of bacteria to mineralize *n*-alkanes has been examined by monitoring the presence of the *alkB*, *alkM*, and *alkB/alkB1* genes, encoding the alkane hydroxylase classified into Group I, II, and III, responsible for the hydroxylation of medium-chain-length *n*-alkanes (C₆–C₁₂), long-chain *n*-alkanes (>C₁₂), and unknown for substrate specificity, respectively (Kohno et al., 2002). The ability of bacteria to mineralize aromatic hydrocarbons has been examined by monitoring the presence of the: (1) *todC1* gene that encodes for the large subunit of terminal dioxygenase, one of the three components of toluene dioxygenase, the first enzyme in the toluene degradation pathways (Whyte et al., 1996); (2) *xyIM* gene that encodes toluene/xylene monooxygenase, responsible for the first step of toluene and *o*-xylene degradation (Baldwin et al., 2003; Bühler et al., 2000); (3) gene that encodes for PAH dioxygenase, responsible for the first step of polyaromatic compounds degradation (Meyer et al., 1999); and (4) the gene that encodes catechol 2,3-dioxygenase, which catalyzes the cleavage of catechols within the lower pathway of aromatic hydrocarbon metabolism (Mesarch et al., 2000).

Efflux-mediated resistance to several antimicrobial agents has been reported in a variety of bacterial species, and a number of efflux determinants have been cloned and sequenced (Poole, 2005). Phylogenetic analysis of RND transporters had showed that they could be separated into seven distinct families, including three hydrophobic/amphiphilic efflux families: the largely Gram-negative bacterial efflux family (HAE1), the Gram-positive bacterial putative efflux family

(HAE2), and the largely archaeal putative efflux family (HAE3) (Tseng et al., 1999). The HAE1 family is largely predominant and includes the well known drug efflux pumps of Gram-negative bacteria with very broad substrate specificity (Meguro et al., 2005). The AcrAB efflux system is composed of the transporter AcrB of the RND family, and the periplasmic accessory protein AcrA. The outer membrane protein TolC is likely to work together with AcrAB (Kobayashi et al., 2001; Nikaido and Zgurskaya, 2001) and it protects bacteria against a variety of toxic substances (Aono et al., 1998; Kobayashi et al., 2001; Nikaido and Zgurskaya, 2001). AcrB of *Escherichia coli* is the best studied member of the HAE1 family (Poole, 2001; Pos, 2009). Homologues of *acrAB* are apparently widespread among Gram-negative bacteria and were found in various genera (Nikaido, 1996; Nikaido and Zgurskaya, 2001; Poole, 2005; Singh et al., 2010; Yang et al., 2008).

Gram-positive and Gram-negative bacteria isolated in the present study were subsequently examined for the presence of known catabolic genes (i.e., alkane hydroxylase Group I, II and III, toluene dioxygenase, toluene/xylene monooxygenase, PAH dioxygenase, catechol 2,3-dioxygenase) and also for the presence of some transporter genes (HAE1, *acrAB*). The expected PCR products sizes were 185 bp, 271 bp, 330 bp, 560 bp, 475 bp, 900 bp, and 238 bp for alkane hy-

droxylase Group I, II, III, toluene dioxygenase, toluene/xylene monooxygenase, PAH dioxygenase, and catechol 2,3-dioxygenase, respectively. The expected PCR products sizes were 550 bp and 471–494/537–560 bp for HAE1, and *acrAB* transporter genes, respectively.

The PCR analysis of Gram-negative bacteria using ALK primers indicated that four strains (*Shewanella* sp. Po₆, Po₁₃, *Aeromonas* sp. Po₈, *Pseudomonas* sp. Po₁₁) possessed alkane hydroxylase Group I gene, one strain (*Shewanella* sp. Po₆) possessed the alkane hydroxylase Group II gene and four strains (*Aeromonas* sp. Po₈, *Pseudomonas* sp. Po₁₀, Po₁₁, Po₁₄) possessed the alkane hydroxylase Group III gene (Table 5). For the Gram-positive bacteria five strains (*Bacillus* sp. Po₂, *Lysinibacillus* sp. Po₃, Po₇, *Rhodococcus* sp. Po₄, Po₅) possessed only the alkane hydroxylase Group III gene.

The PCR analysis of Gram-negative bacteria using todC1, TOL, 23CAT primers indicated that four strains (*Pseudomonas* sp. Po₉, Po₁₀, Po₁₁, *Klebsiella* sp. Po₁₂) possessed the toluene dioxygenase gene, three strains (*Pseudomonas* sp. Po₉, Po₁₁, Po₁₄) possessed the toluene/xylene monooxygenase gene and three strains (*Aeromonas* sp. Po₈, *Pseudomonas* sp. Po₉, *Shewanella* sp. Po₁₃) possessed the gene encoding for catechol 2,3-dioxygenase (Table 5). Gram-positive bacterial strains did not amplify todC1, TOL or 23CAT

Table 5. Screening of Gram-positive and Gram-negative bacteria for the presence of catabolic genes and transporter genes.

Bacterial strain	PCR product of:									
	Catabolic genes							Transporter genes		
	Alkane hydroxylase			Toluene dioxygenase	Toluene/xylene monooxygenase	PAH dioxygenase	Catechol 2,3-dioxygenase	HAE1	acrAB	
	ALK-1f	ALK-2f	ALK-3f	todC1-f	TOL-f	ISPGRL1B	23CAT-f	A24f2	acrA-f	
	ALK-1r	ALK-2r	ALK-3r	todC1-r	TOL-r	ISPGRR1B	23CAT-r	A577r2	acrB-r	
	T _a = 43°C	T _a = 43°C	T _a = 43°C	T _a = 53°C	T _a = 53°C	T _a = 60°C	T _a = 56°C	T _a = 50°C	T _a = 50°C	
<i>Bacillus</i> sp. Po ₁	–	–	–	–	–	–	–	–	–	
<i>Bacillus</i> sp. Po ₂	–	–	+	–	–	–	–	–	–	
<i>Lysinibacillus</i> sp. Po ₃	–	–	+	–	–	–	–	–	–	
<i>Rhodococcus</i> sp. Po ₄	–	–	+	–	–	–	–	–	–	
<i>Rhodococcus</i> sp. Po ₅	–	–	+	–	–	–	–	–	–	
<i>Shewanella</i> sp. Po ₆	+	+	–	–	–	–	–	+	+	
<i>Lysinibacillus</i> sp. Po ₇	–	–	+	–	–	–	–	–	–	
<i>Aeromonas</i> sp. Po ₈	+	–	+	–	–	–	+	–	+	
<i>Pseudomonas</i> sp. Po ₉	–	–	–	+	+	–	+	+	–	
<i>Pseudomonas</i> sp. Po ₁₀	–	–	+	+	–	–	–	+	+	
<i>Pseudomonas</i> sp. Po ₁₁	+	–	+	+	+	–	–	+	+	
<i>Klebsiella</i> sp. Po ₁₂	–	–	–	+	–	–	–	+	+	
<i>Shewanella</i> sp. Po ₁₃	+	–	–	–	–	–	+	–	+	
<i>Pseudomonas</i> sp. Po ₁₄	–	–	+	–	+	–	–	+	+	

f = forward PCR primer; r = reverse PCR primer; T_a = the annealing temperature.

fragments. None of the isolated bacterial strains showed positive results for the gene that encodes for PAH dioxygenase. Additional fragments larger or smaller than expected were obtained for some bacterial strains when ALK-1, ALK-3, todC1, TOL and 23CAT primers were used.

According to the literature, the use of specific primers to amplify catabolic genes is a very useful tool for the detection of a broader subset of environmentally important hydrocarbon-degrading bacteria (Márquez-Rocha et al., 2005; Mesarch et al., 2000; Whyte et al., 1996). Nevertheless, it is true that the presence of a single gene does not ensure that the entire catabolic pathway will be present or that these genes will be expressed (Mesarch et al., 2000). Different specific primers for other genes involved in hydrocarbon catabolic pathways will be used to determine whether multiple pathway genes are present in isolated bacterial strains.

The PCR analysis of the Gram-positive and Gram-negative bacteria using A24-A577 and *acrAB* primers indicated that six strains (*Shewanella* sp. Po₆, *Pseudomonas* sp. Po₉, Po₁₀, Po₁₁, Po₁₄, *Klebsiella* sp. Po₁₂) possessed the HAE1 transporter gene (Table 5, Fig. 2c) and seven strains (*Shewanella* sp. Po₆, Po₁₃, *Aeromonas* sp. Po₈, *Pseudomonas* sp. Po₁₀, Po₁₁, Po₁₄, *Klebsiella* sp. Po₁₂) possessed the *acrAB* transporter gene (Table 5). As anticipated, Gram-positive bacterial strains did not amplify HAE1 or *acrAB* fragments. However, additional fragments larger or smaller than expected were also obtained for some Gram-positive and Gram-negative bacterial strains when A24-A577 and *acrAB* primers were used.

Isolation of new bacterial strains able to grow in crude oil as the single carbon source is attractive from the environmental point of view. However, many bacterial strains have already been identified as oil-degrading bacteria, many of them belonging to other genera than those isolated by us. This study provides an insight for a better understanding of the adaptation of bacteria inhabiting polluted environments to environmental fluctuations of nutrients, and for developing and implementing adequate bio-strategies for the remediation of oily sludge-contaminated environments. As we mentioned previously, isolated bacterial strains were tolerant to saturated, monoaromatic, and polyaromatic hydrocarbons, but also resistant to different antimicrobial agents. Bacteria sometimes exhibit cross-protection mechanisms when they are exposed

to different stressors (e.g., hydrocarbons) enhancing their resistance to different toxic compounds (e.g., antibiotics). However, in this study the presence of hydrophilic antibiotics (i.e., ampicillin or kanamycin) in the culture medium had no effects on Gram-positive or Gram-negative bacteria resistance to toxic compounds (i.e., dyes, detergents, or hydrocarbons). The results indicated that Gram-negative bacteria are in general less sensitive to toxic compounds than Gram-positive bacteria. Gram-negative bacteria possessed between two and four catabolic and transporter genes, compared with Gram-positive bacteria which possessed one catabolic gene and no transporter genes. These could explain the observed differences in the resistance of Gram-positive and Gram-negative bacteria to toxic compounds. The further characterization of these hydrocarbon-tolerant bacteria will provide the knowledge required for their use in biotechnological applications. Further studies will be carried out, as well as genomic DNA screening by PCR for the presence of other catabolic genes involved in known hydrocarbon biodegradative pathways, and for the presence of other efflux pumps of the RND family.

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