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유기용매에 의하여 유발된 *Aeromonas hydrophila* IBB_{Ct4}의 변형

Mihaela Marilena Lăzăroaie*
Center of Microbiology, Institute of Biology, Romanian Academy, 296 Spl. Independentei St, 060031, PO 56-53, Bucharest, Romania
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Modifications Induced by Organic Solvents to *Aeromonas hydrophila* IBB_{Ct4}

Mihaela Marilena Lăzăroaie*
Center of Microbiology, Institute of Biology, Romanian Academy, 296 Spl. Independentei St, 060031, PO 56-53, Bucharest, Romania
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요 약. 유기용매들에 대한 내성을 갖고 분해할 수 있는 능력을 가진 박테리아가 해수에서 단리되었지만, 해양조건에서 유기용매가 분해될 때 그들의 역할은 잘 알려져 있지 않았다. 콘스탄차 해수에서 단리된 *Aeromonas hydrophila* IBB_{Ct4}는 여러 다른 종류의 유기용매들에 대하여 내성을 갖고 분해할 수 있는 능력을 가지고 있다. 옥탄올-헥산 혼합물에서 2.64 - 3.17 사이의 분배계수지수 ($\log P_{ow}$)를 가진 톨루엔, 크실렌 이성질체들 및 에틸벤젠은, 3.69 - 4.39 사이의 $\log P_{ow}$를 가진 프로필벤젠, n-헥산 및 n-헵탄보다 이들 박테리아에 더 독성이 있었다. *Aeromonas hydrophila* IBB_{Ct4}에 유기용매들을 처리하였을 때 분자수준에서 세포의 변형이 유발되었다. 다른 유기용매들에 의하여 분자수준에서의 세포변화가 다르게 나타난다는 것을 밝힌 이 연구는 배양 조건에서 유기용매에 대한 박테리아 세포들의 반응이 복잡하다는 것을 보여 주고 있다.

주제어: *Aeromonas hydrophila*, 유기용매, 변형

ABSTRACT. Many bacteria that are capable to tolerate and degrade organic solvents have been isolated from seawater. However, their roles in the biodegradation of organic solvents in the marine environment have remained unknown. *Aeromonas hydrophila* IBB_{Ct4}, isolated from Constanta seawater, was able to tolerate and degrade different organic solvents. Toluene, styrene, xylene isomers, ethylbenzene, with the logarithm of the partition coefficient in octanol-water mixture ($\log P_{OW}$) between 2.64 and 3.17, were more toxic for bacterial cells, compared with propylbenzene, *n*-hexane, *n*-heptane, with $\log P_{OW}$ between 3.69 and 4.39. There were revealed cellular and molecular modifications induced by organic solvents to *Aeromonas hydrophila* IBB_{Ct4}. The study of cellular and molecular modifications induced by different organic solvents showed a complex response of bacterial cells to the presence of organic solvents in the culture media.

Keywords: *Aeromonas hydrophila*, Organic solvents, Modification

INTRODUCTION

Oil and gasoline spills contain relatively high concentrations of organic solvents. There are various types of organic solvent molecules, such as alkanes, alkenes, cycloalkanes, cycloalkenes, and aromatics.[1] Although the release of organic solvents is restricted by legislation, a certain amount of such compounds have already reached the environment and need to be eliminated. Upon the release of organic solvents in the marine environment, their transformation starts immediately, and microbial

degradation is considered to be a major route for the breakdown of such toxic components.[2,12] Organic solvents with the logarithm of the partition coefficient in octanol-water mixture (log $P_{O/W}$) between 1.5 and 4 are compounds with high toxicity for the bacterial cells, so that, as a result of their partition in lipid bilayer, they produce significant modifications in the membrane structure and functions.[3] This, together with the loss of lipids and proteins, leads to irreversible damage, resulting in the death of the cell.[3,9,10,13,14] Nevertheless, bacteria that can grow under these conditions have been found, suggesting that they may have the ability to resist to high concentrations of organic compounds.[10,15,16]

Although the use of different bacterial groups in bioremediation processes has been widely studied, the ability of native communities of halophilic bacteria to be used for the degradation of pollutants in saline habitats has not been studied in a systematic way, and only a few studies have reported the biotechnological potential of this group of extremophiles in the decontamination of saline environments.[16-18] The aim of this study was to collect data which can shed light on cellular and molecular modifications induced by different toxic organic solvents (*n*-hexane, *n*-heptane, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) to moderately halophilic bacteria *Aeromonas hydrophila* IBB_{Ch4}, for developing and implementing adequate bio-strategies for the remediation of marine polluted environments.

EXPERIMENTAL SECTION

Bacterial strain. Isolation of IBB_{Ch4} bacterial strain from seawater (Constanta County, Romania) was carried out on ASW[16] medium (artificial seawater containing NaCl 2% w/v), using the enriched cultures method, with organic solvents as single carbon source. The use of ASW medium with organic solvents as single carbon source allowed the selective development of IBB_{Ch4} solvent-tolerant bacterial strain. For further characterization of bacterial strain several physiological and biochemical tests were performed: Gram reaction, morphology, endospore formation, mobility, respirator type, pigments production, growth on Coomassie brilliant blue R250 agar (CBB agar), growth on medium with NaCl, catalase production, oxidase production, nitrates reduction, indole production, D-glucose fermentation, L-arginine dihydrolase and urease production, esculin and gelatin hydrolysis, β-galactosidase production, and assimilation of different substrates such as D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, phenylacetic acid. The taxonomic affiliation of bacterial strain to *Aeromonas* genus was determined based on its phenotypic characteristics and also based on the G+C content of the bacterial chromosome.[19]

Tolerance and degradative capacity of *Aeromonas hydrophila* IBB_{Ch4} to organic solvents (*n*-hexane, *n*-heptane, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene). Bacterial cells in the exponential phase of growth (10^7 cells mL^{-1}) were cultivated on liquid LB_{ASW}[16] medium (control) or liquid ASW[16] medium, added with 1% (w/v) yeast extract (control) and on the same media in the presence of 1%, 5% (v/v) organic solvents. Flasks were sealed and incubated 24 hours at 28°C on a rotary shaker (150-200 rpm). The growth of the bacterial strain was determined by spetrophotometric measurement of the optical density (OD_{660nm}). Bacterial cells in the exponential phase of growth were also plated on solid LB_{ASW} medium (control) or solid ASW medium added with 1% (w/v) yeast extract (control) and on the same media with organic solvents, supplied in the vapor phase. Petri plates were sealed and the formation of organic solvents-resistant bacterial colonies on the agar was determined after 24 hours incubation at 28 °C.

Cellular and molecular modifications induced by organic solvents (*n*-hexane, *n*-heptane, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) **on *Aeromonas hydrophila* IBB_{Ch4}.** Bacterial cells in the exponential phase of growth (10^7 cells mL^{-1}) were cultivated on liquid LB_{ASW}[16] medium (control) and on the same medium in the

presence of 1%, 5% (v/v) organic solvents. Flasks were sealed and incubated 24 hours at 28 °C on a rotary shaker.

Modifications induced by organic solvents to cells viability. Serial dilutions of culture liquid were spread on agar LB_{ASW} medium using the method of Ramos *et al.*[8] and the number of viable cells (CFU mL^{-1}) was determined.

Modifications induced by organic solvents to cell wall hydrophobicity. Bacterial adhesion to organic solvents was determined using the method of Rosenberg *et al.*[20] The bacterial adhesion to organic solvents was also studied on wet mount with the optical microscope.

Modifications induced by organic solvents to lipids. Lipids were extracted with chloroform-methanol (2:1) mixture using the method of Benning and Somerville.[21] The samples were spotted onto 20×20cm Silica gel 60 TLC aluminium sheets (Merck), and the separation was performed using chloroform-methanol-acetic acid-water (85:22.5:10:4 v/v/v/v) mixture as mobile phase. 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 phospholipids was done based on their motilities (R_f) and their comparison with those of phospholipid standards (Sigma-Aldrich, Supelco).

Modifications induced by organic solvents to DNA. The bacterial cells were lysed with TE buffer (10 mM Tris-HCl, 1 mM EDTA) and the DNA was extracted with phenol-chloroform-isoamilic alcohol (25:24:1 v/v/v) mixture, precipitated with ethanol and resuspended in TE buffer. DNA was visualized after separation on 0.8% (w/v) TBE (Tris-Borate-EDTA) agarose gel by staining with fast blast DNA stain (Bio-Rad). DNA content and purity were measured by the method of Sambrook *et al.*[22] DNA concentration of each variant was adjusted to 100 μg mL^{-1} for spectral comparison (OD_{260nm}-OD_{350nm}). For PCR amplification, 5 μL of DNA extract was added to a final volume of 50 μL reaction mixture, containing: 5×GoTaq flexi buffer, $MgCl_2$, dNTP mix, primers (A24f2 5'-CCSRTITTYGCITGGGT-3', A577r2 5'-SAICCARAIRCGCATSGC-3'), GoTaq DNA polymerase (Promega). PCR was performed with a C1000 thermal cycler (Bio-Rad). PCR program consisted in initial denaturation for 5 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, anneling at 48.0 to 54.3 °C for 1 min, and extension step at 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR were performed in duplicate. After separation on 1.6% (w/v) TBE agarose gel and staining with fast blast DNA stain (Bio-Rad) the amplified fragments were analyzed.

The influence of organic solvents to rhodamine 6G accumulation in bacterial cells. Bacterial cells were spotted on agar LB_{ASW} medium (control) and on the same medium with rhodamine 6G (1, 10 μg mL^{-1}). Rhodamine 6G accumulation in bacterial cells was observed under UV light after 24 hours of incubation at 28 °C.

Reagents. *n*-hexane (96% pure), toluene (99% pure), propylbenzene (98% pure), xylene isomers (99% pure), styrene (99% pure) were obtained from Merck (E. Merck, Darmstadt, Germany), *n*-heptane (97% pure), ethylbenzene (98% pure) were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Other reagents used were procured from Merck, Sigma-Aldrich, Difco Laboratories (Detroit, Michigan, USA), Promega (Promega GmbH, Mannheim, Germany), bioMérieux (Marcy-l'Etoile, France) or Bio-Rad Laboratories (Alfred Nobel Dr., Hercules, CA, USA).

RESULTS AND DISCUSSION

Extensive petroleum hydrocarbon exploration activities often result in the pollution of the environment, which could lead to disastrous consequences for the biotic and abiotic components of the ecosystem if not restored. Remediation of petroleum-contaminated system could be achieved by either physicochemical or biological methods. However, the attendant negative consequences of the physicochemical approach are currently directing greater attention to the exploitation of the biological alternatives.[23] Many bacteria that are capable to tolerate and degrade organic solvents

have been isolated from polluted and unpolluted seawater however their roles in the biodegradation of organic solvents have remained unknown. These microorganisms are of interest for the bioremediation of sites that are heavily polluted with organic compounds, for the biotransformation of low-solubility chemicals in water, and for the construction of robust biosensor strains for the *in situ* detection of pollutants.[4,13,24]

IBB_{Ct4} bacterial strain was Gram-negative, non-spore-forming rods. Optimal growth temperature was 28 °C. Cells occurred singly. Motile. Aerobic, facultative anaerobic. On solid LB_{ASW} medium, colonies were cream. On solid CBB medium, colonies were dark blue. Bacterial strain grew well in a medium containing up to 10% (w/v) NaCl. Pyocyanine and pyoverdine/fluoresceine-negative. Catalase-negative and oxidase-positive. Nitrate was reduced to nitrite but nitrite was not reduced. Indole, β-galactosidase and L-arginine dihydrolase were produced. Acid was produced from D-glucose. Urease was not produced. Esculin and gelatin were hydrolysed. D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, malic acid and trisodium citrate were used as sole carbon and energy sources. Adipic acid and phenylacetic acid were not used as single carbon and energy sources. The identification result for strain IBB_{Ct4} with G+C content 61.5 mol% corresponded to *Aeromonas hydrophila*. Aeromonads belong to the family *Aeromonadaceae* within the Gamma subclass of the *Proteobacteria*. According to the literature (Seshadri *et al.*, 2006), *Aeromonas* spp. are ubiquitous bacteria found in a variety of aquatic environments worldwide, including bottled water, chlorinated water and also heavily polluted waters.[25,26]

Tolerance and degradative capacity of *Aeromonas hydrophila* IBB_{Ct4} to organic solvents. *Aeromonas hydrophila* IBB_{Ct4} was able to tolerate and degrade 1% and 5% (v/v) organic solvents such as *n*-hexane, *n*-heptane, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene (*Table* 1). The toxicity of organic solvents is generally in inverse correlation with the logarithm of the partition coefficient of the organic solvent in octanol-

Table 1. Tolerance and degradative capacity of *Aeromonas hydrophila* IBB_{Ct4} to organic solvents

Variant:	log P_{OW}[1]	Bacterial strain growth on liquid medium[2] with organic solvents (v/v)				Bacterial strain growth on solid medium[3] with organic solvents in vapor phase	
		LB_{ASW} medium		ASW medium			
		1% (v/v)	5% (v/v)	1% (v/v)	5% (v/v)	LB_{ASW} medium	ASW medium
Control	-	1.289	1.289	0.953	0.953	100%	100%
n-Hexane	3.86	1.090	0.795	0.382	0.364	100%	50%
n-Heptane	4.39	0.969	0.712	0.286	0.212	100%	50%
Toluene	2.64	0.116	0.011	0.055	0.014	10%	0.01%
Styrene	2.86	0.135	0.021	0.080	0.008	10%	0.01%
o-Xylene	3.09	0.308	0.059	0.152	0.047	10%	0.01%
m-Xylene	3.14	0.205	0.089	0.083	0.031	10%	0.01%
p-Xylene	3.14	0.214	0.079	0.155	0.043	10%	0.01%
Ethylbenzene	3.17	0.335	0.078	0.185	0.047	10%	0.01%
Propylbenzene	3.69	0.402	0.198	0.207	0.082	50%	25%

Legend: [1] = logarithm of the partition coefficient of the organic solvent in octanol-water mixture; [2] = the growth of the bacterial strain on liquid medium was determined by spectrophotometric measurement of the optical density (OD_{660nm}) 24 hours after organic solvents shock; [3] = the growth on solid medium was estimated by determining the formation of resistant bacterial colonies and the solvent resistance is represented by the frequency of colony formation, with that observed in the absence of any organic solvent taken as 100%.

water mixture (log P_{OW}), thus solvents with lower log P_{OW} show higher toxicity to microorganisms.[3,10,13] However, hydrocarbon toxicity depends not only on the inherent toxicity of the compound, but also on the intrinsic tolerance of the bacterial strain.[10] *n*-hexane, *n*-heptane, propylbenzene, with log P_{OW} between 3.69 and 4.39, were less toxic (OD_{660} = 0.198-1.090) for *Aeromonas hydrophila* IBB_{Ct4} cells, compared with toluene, styrene, xylene isomers and ethylbenzene (OD_{660} = 0.011-0.335), with log P_{OW} between 2.64 and 3.17. The assessment of organic solvents degradation capacity for *Aeromonas hydrophila* IBB_{Ct4} indicated that the strain was able to use as single source of carbon 1% and 5% (v/v) *n*-hexane, *n*-heptane, propylbenzene (OD_{660} = 0.082-0.382), and only 1% (v/v) toluene, styrene, xylene isomers or ethylbenzene (OD_{660} = 0.055-0.185) (*Table* 1). The growth of *Aeromonas hydrophila* IBB_{Ct4} on agar media supplied with organic solvents in the vapor phase was 25-100% in the presence of *n*-hexane, *n*-heptane, propylbenzene, and 0.01-10% in the presence of toluene, styrene, xylene isomers and ethylbenzene.

Concerning the tolerance to organic solvents, a number of elements have been suggested as being involved in the response to these toxic compounds: metabolism of the toxic organic solvents, which may contribute to their transformation into non-toxic compounds, rigidification of the cell membrane via alteration in the composition of phospholipids, and efflux of the toxic compounds in an energy-dependent process.[10,13] Although the metabolism of the toxic compounds can help to reduce their toxicity, two lines of evidence suggest that it is of minor importance. A number of microorganisms tolerant to different organic solvents cannot metabolize the toxic compound, e.g. *Escherichia coli* strains are tolerant to 1% (v/v) *n*-hexane, but do not use (or biotransform) this compound.[27] and different *Pseudomonas* strains are tolerant up to 50% (v/v) toluene but did not use this compound as a carbon source.[28] *Pseudomonas putida* DOT-T1E is a toluene-tolerant strain that degrades and also uses this chemical via the toluene-dioxygenase pathway.[29] This therefore establishes a neat difference between the metabolism of a chemical and the tolerance to it. Instead, lipid membrane ridigification in response to toxic compounds and efflux of the chemicals are key elements in solvent tolerance, although their role has not been investigated in the same extent.[13,14]

Cellular and molecular modifications induced by organic solvents on *Aeromonas hydrophila* IBB_{Ct4}. In order to establish the adaptation mechanisms that protect *Aeromonas hydrophila* IBB_{Ct4} cells against toxic effects of organic solvents, I determined the modification induced on cellular and molecular level 24 hours after organic solvents shock, compared with control (cells incubated without solvents).

Modifications induced by organic solvents to cells viability. Organic solvents with higher log P_{OW} (3.69 and 4.39), such as *n*-hexane, *n*-heptane, propylbenzene were binding less abundantly to viable bacterial cells, being less toxic for them, while solvents with lower log P_{OW} (2.64-3.17), such as toluene, styrene, xylene isomers and ethylbenzene were binding more abundantly to viable bacterial cells, being more toxic for them (*Table* 2). When 1% (v/v) organic solvents were added to culture liquid, the survival rate were between 10^3 and 10^9 CFU mL^{-1}, while when 5% (v/v) *n*-hexane, *n*-heptane, xylene isomers, ethylbenzene or propylbenzene were added to culture liquid, the survival rate were between 10 and 10^8 CFU mL^{-1}. When 5% (v/v) toluene or styrene were added to culture liquid, the survival rates decreased significantly, below the detection limit of the experiment. Against the idea that the toxicity of a second phase of an organic solvent can be inferred from its hydrophobicity, reflected by its log P_{OW},[30] it was observed that some marine toluene tolerant bacteria were unable to grow in the presence of some organic solvents with log P_{OW} values higher than toluene.[16] This was also previously observed for *Pseudomonas putida* DOT-T1E, which thrived in the presence of toluene (log P_{OW} = 2.64), but it was unable to do so in the presence of 1-octanol (log P_{OW} = 2.93). Although 1-octanol did not

Table 2. The modifications of cell viability and cell hydrophobicity of *Aeromonas hydrophila* IBB_{Cl4} after organic solvents shock

Variant	log P_{OW}[a]	Bacterial cell viability[b] (CFU mL^{-1}) after organic solvents shock:		Bacterial cell hydrophobicity[c] (% BATS) after organic solvents shock:	
		1% (v/v)	5% (v/v)	1% (v/v)	5% (v/v)
Control	-	3.6×10^{11}	3.6×10^{11}	-	-
n-Hexane	3.86	5.0×10^{9}	2.4×10^{8}	18.04	8.64
n-Heptane	4.39	6.3×10^{9}	3.7×10^{8}	16.68	7.70
Toluene	2.64	2.3×10^{3}	0	2.17	0.20
Styrene	2.86	1.5×10^{4}	0	3.70	0.13
o-Xylene	3.09	4.5×10^{5}	1.8×10^{2}	6.20	1.25
m-Xylene	3.14	4.0×10^{4}	1.5×10	7.75	1.34
p-Xylene	3.14	4.2×10^{4}	1.3×10	7.87	1.45
Ethylbenzene	3.17	5.4×10^{5}	3.3×10^{2}	8.39	4.40
Propylbenzene	3.69	4.1×10^{7}	3.0×10^{5}	16.30	6.30

Legend: [a] = logarithm of the partition coefficient of the organic solvent in octanol-water mixture; [b] = serial dilutions of culture liquid were spread on agar medium and the number of viable cells (CFU mL^{-1}) was determined 24 hours after organic solvents shock; [c] = the decrease of the turbidity in aqueous phase in the presence of organic solvents (% BATS) was determined 24 hours after organic solvents shock.

decrease cell viability, it completely impeded growth of the culture. To explain these findings, the different partition of the hydrocarbon in the membrane was invoked.[31]

Modifications induced by organic solvents to cell wall hydrophobicity. The affinity of bacterial cells for hydrophobic interfaces is an important property that directly affects the efficiency of various bioprocesses, such as bioremediation and waste treatment, using whole microbial cells. Although the outcome of bacterial adhesion to hydrocarbon tests (BATH) is affected not only by hydrophobic interactions but also by van der Waals and electrostatic interactions, in this test method, the behavior of microbial cells in a two-liquid-phase system and the interaction of the cells with an organic phase, including the affinity of the cells for the organic surface, can be directly evaluated.[32] Although the organic solvents 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 the tubes containing the aqueous phase (cell suspension) and the organic phase (solvents). It was observed that the bacterial cells adhere to the organic solvents microdroplets formed as a result of mechanical dispersion, which are stable, causing the decrease of the turbidity in the aqueous phase. *Aeromonas hydrophila* IBB_{Cl4} cells presented higher hydrophobicity (6.30-18.04%) in the presence of *n*-hexane, *n*-heptane and propylbenzene, compared with cell hydrophobicity (0.13-8.39%) in the presence of toluene, styrene, xylene isomers and ethylbenzene (*Table* 2). The low hydrophobicity of the cell wall represents a defensive mechanism, which keep away the organic solvents molecules from the cell surface, preventing accumulation of the toxic compounds in high concentrations in the bacterial cell membranes, fact confirmed by the optical microscope observations (data not shown).

Modifications induced by organic solvents to lipids. The initial stage of damages caused by many solvents, when bacterial cells are exposed to organic solvents, consists in the binding and penetration of the lipid bilayer.[3] As a consequence, membrane fluidity is affected, and bacteria undertake appropriate responses to diminish its disruptive effect. This is achieved by readjusting its fluidity, primarily by altering the composition of

the lipid bilayer. This response is apparent in almost all membrane constituents. The compensation mechanisms resemble some of those observed in response to physical and chemical changes imposed by the environment. The major responses to solvent exposure, with regard to membrane composition are: change in fatty acid composition, *cis/trans* fatty acid isomerization, saturated/unsaturated fatty acid regulation and change in phospholipid headgroups and the rate of their synthesis. The change in phospholipid headgroups is a less well studied phenomenon, and few data on it are available.[8,10,13,33-35] In *Haloarcula* strains OHF-1, OHF-2 and *Haloarcula argentinensis* in the presence of *n*-decane the levels of phosphatidylglycerol (PG) decreased considerably, while those of phosphatidylglycerosulfate (PGS) and phosphatidylglycerophosphate methyl ester (PGP-Me) increased.[35]

The thin-layer chromatography (TLC) studies revealed the existence of some differences between phospholipids motilities and phospholipid headgroups composition extracted from *Aeromonas hydrophila* IBB_{Ct4} cells incubated without organic solvents (control) and those extracted from cells 24 hours after 1% (v/v) organic solvents shock (*Figure* 1a).

The phospholipids found, based on their motilities (R_f), in *Aeromonas hydrophila* IBB_{Ct4} cells (control) were lysophosphatidylcholine (LPC with R_f 0.13), phosphatidylcholine (PC with R_f 0.16), unknown phospholipid (UNK with R_f 0.18), phosphatidylethanolamine (PE with R_f 0.32), phosphatidylglycerol (PG with R_f 0.46), phosphatidylinositol (PI with R_f 0.57), and cardiolipin/diphosphatidylglycerol (CL with R_f 0.97). The phospholipids found in *Aeromonas hydrophila* IBB_{Ct4} cells after organic solvents shock were lysophosphatidylcholine (LPC with R_f 0.14-0.18), phosphatidylcholine (PC with R_f 0.17-0.23), unknown phospholipid (UNK with R_f 0.19-0.27), phosphatidylethanolamine (PE with R_f 0.33-0.40), phosphatidylglycerol (PG with R_f 0.49-0.58), phosphatidylinositol (PI with R_f 0.58-0.70), and cardiolipin/diphosphatidylglycerol (CL with R_f 0.93-0.98). Therefore, different solvent-

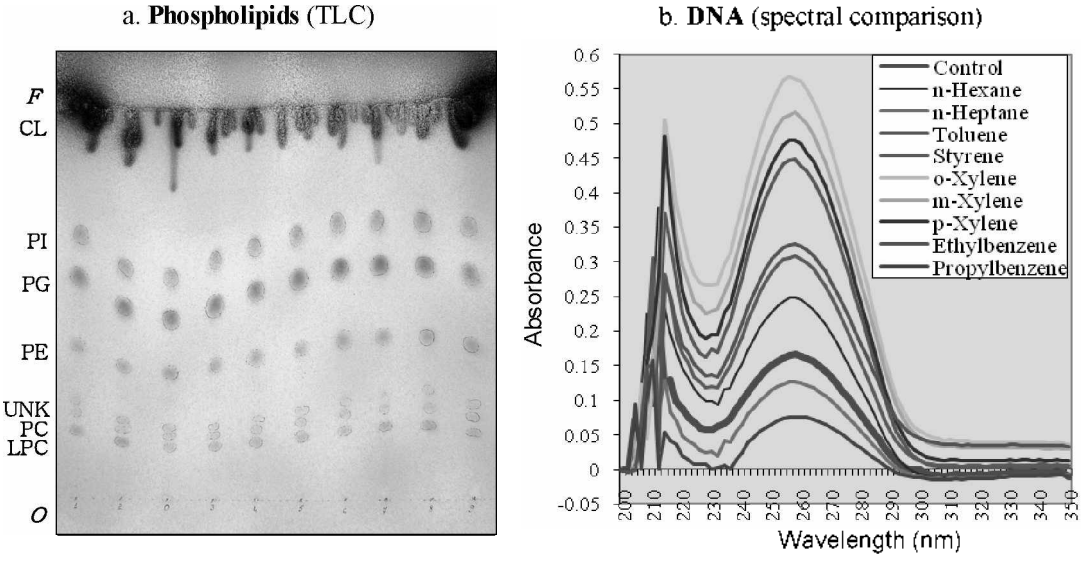

Figure 1. The lipid profile (**a.**) and DNA (**b.**) modifications of *Aeromonas hydrophila* IBB_{Ct4} after organic solvent shock. Panel **a**. Control (lane ***C***); *n*-hexane (lane ***1***); *n*-heptane (lane ***2***); toluene (lane ***3***); styrene (lane ***4***); *o*-xylene (lane ***5***); *m*-xylene (lane ***6***); *p*-xylene (lane ***7***); ethylbenzene (lane ***8***); propylbenzene (lane ***9***); origin (***O***), solvent front (***F***), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), unknown phospholipids (UNK), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), cardiolipin (CL). Panel **b**. DNA concentration of each variant was adjusted to 100 μg mL^{-1} for spectral comparison (OD_{200nm}-OD_{350nm}).

tolerant strains seem to have developed different strategies for changing phospholipid headgroup composition to increase membrane rigidity and, in this way, to overcome the damaging effects of solvents.[3,8,10,13,33-37]

Modifications induced by organic solvents to DNA. There were also observed, by spectral comparison, differences between the DNA extracted from *Aeromonas hydrophila* IBB_{Ch} cells incubated without organic solvents (control) and those extracted from cells 24 hours after 1% (v/v) organic solvents shock (*Figure* 1b). The spectrum of the DNA extracted from bacterial cells incubated with *n*-hexane, styrene, ethylbenzene, toluene and xylene isomers was above than the DNA spectrum of the control. The spectrum of the DNA extracted from bacterial cells incubated with *n*-heptane and propylbenzene was below than the DNA spectrum of the control. The hydrocarbons often produce lesions on DNA level (in purine base).[38] By DNA sequence analysis, activated aromatic hydrocarbons have been found to induce G-C and T-A base pair insertions and deletions. Aromatic hydrocarbons are metabolically activated in cells to yield highly reactive bay region dihydrodiol epoxide derivatives.[38] Dihydrodiol epoxides are electrophilic and can effectively attack DNA, forming covalently linked bulky adducts on DNA bases. These adduct cause structural changes in DNA, thus leading to disruption of normal cellular functions, such as transcription and replication. Furthermore, if not repaired, damaged nucleotides can result in mutations during replication.[38]

According to the literature,[39] bacteria are equipped with some device which protects the cells from different organic solvents. The most likely candidates for such a device are RND-type (resistance-nodulation-cell division) efflux pumps, found in a wide variety of Gram-negative bacteria, including *Aeromonas hydrophila*, since the outer membrane protein seems to be a pump component.[26,39-42] An RND efflux pump comprises of three subunits: an RND transporter, a membrane fusion protein and an outer-membrane protein.[43] To determine if

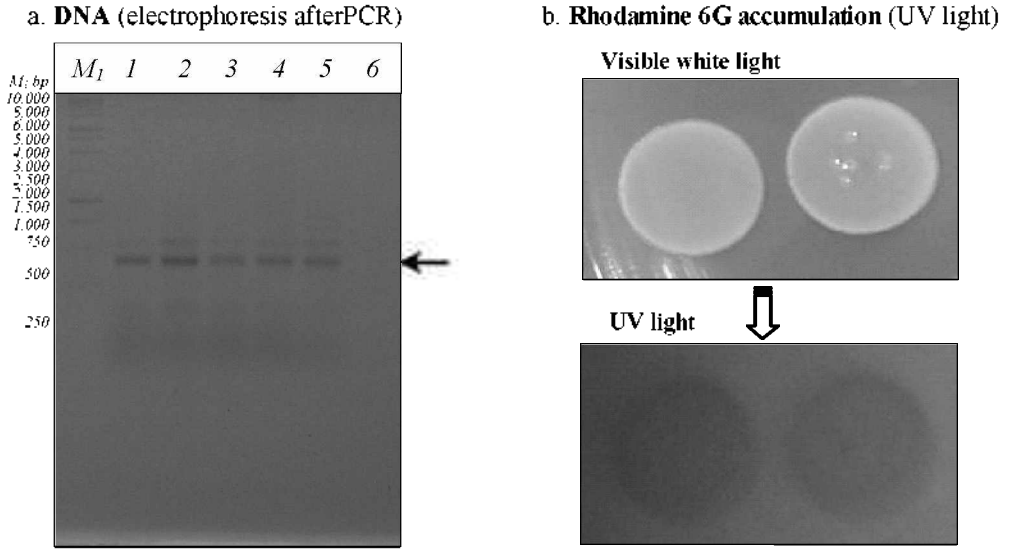

Figure 2. PCR amplification of HAE1 fragment using as template the DNA extracted from *Aeromonas hydrophila* IBB_{Ch} (panel **a.**) and rhodamine 6G accumulation in *Aeromonas hydrophila* IBB_{Ch} cells (panel **b.**). Panel **a.** 1 kb DNA ladder, Promega (lane ***M1***); annealing at: 48.0 °C (lanes ***1***), 48.7 °C (lanes ***2***), 50.0 °C (lanes ***3***), 51.9 °C (lanes ***4***); 54.3 °C (lanes ***5***); negative control DNA (lane ***6***); the positions of the expected HAE1 fragment (550 bp) are indicated with arrow. Panel **b.** After incubation at 28 °C for 24 h, bacterial colonies were observed under visible white light and UV light.

efflux pumps of RND family were present in new isolated solvent-tolerant bacterial strain, it were used oligonucleotides A24f2 and A577r2 to amplify members of the HAE1 (hydrophobe/amphiphile efflux 1) family of transporters. Using as template the DNA extracted from *Aeromonas hydrophila* IBB_{Ct4}, it was obtained the amplification of the expected 550 bp fragment (*Figure* 2a). It was also obtained the unspecific amplification of another fragment of 800 bp. Further studies will be carried out on this topic. A gradient PCR (50 ± 5°C) will be perform to determine the optimum annealing temperatures of A24f2 and A577r2 primers for *Aeromonas hydrophila* IBB_{Ct4}. The HAE1 family is largely predominant and includes the well known drug efflux pumps of Gram-negative bacteria with very broad substrate specificity.[44]

The influence of organic solvents to rhodamine 6G accumulation in bacterial cells. The existence of solvent efflux pumps in *Aeromonas hydrophila* IBB_{Ct4} cells was confirmed by rhodamine 6G accumulations (1 and 10 μg mL^{-1}) in bacterial cells. Accumulation of rhodamine 6G in *Aeromonas hydrophila* IBB_{Ct4} cells was observed by the fluorescence of rhodamine 6G under UV light (*Figure* 2b). Rhodamine 6G is P-glycoproteins substrates, which mediate the energy-dependent efflux of certain toxic compounds from the bacterial cells. P-glycoprotein has been shown to bind a large number of lipophilic compounds (antibiotics, dyes, polyaromatic hydrocarbons, polycholorinated biphenyls and other toxic compounds) with no apparent structural or functional similarities.[3] The rhodamine 6G accumulation assays showed the existence of some differences between *Aeromonas hydrophila* IBB_{Ct4} cells incubated without organic solvents (control) and bacterial cells incubated 24 hours in the presence of 1% (v/v) organic solvents. It was observed an increase of rhodamine 6G accumulation in *Aeromonas hydrophila* IBB_{Ct4} cells grown in the presence of organic solvents, such as toluene, styrene, xylene isomers and ethylbenzene, compared with the control (data not shown). No significant modifications in rhodamine 6G accumulation were observed in *Aeromonas hydrophila* IBB_{Ct4} cells grown in the presence of *n*-hexane, *n*-heptane, and propylbenzene, compared with the control.

CONCLUSIONS

Aeromonas hydrophila IBB_{Ct4} was able to tolerate and to degrade organic solvents, such as *n*-hexane, *n*-heptane, propylbenzene, and even toluene, styrene, xylene isomers, and ethylbenzene. There were observed cellular and molecular modifications induced by organic solvents to *Aeromonas hydrophila* IBB_{Ct4}. The modifications induced by organic solvents to *Aeromonas hydrophila* IBB_{Ct4} cells differ according to the nature of hydrophobic substrate and also according to the culture conditions. This study is relevant for better understanding the adaptation of bacteria inhabiting marine oil-contaminated environments to environmental fluctuations of nutrients, for developing and implementing adequate bio-strategies for the remediation of marine polluted environments. Further studies will be carried out, as well as the genomic DNA will be screened by PCR for the presence of catabolic genes involved in known organic solvents biodegradative pathways.

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