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Mechanisms Involved In Organic Solvent Resistance in Gram-Negative Bacteria

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***Abstract*—**The high world interest given to the researches concerning the study of moderately halophilic solvent-tolerant bacteria isolated from marine polluted environments is due to their high biotechnological potential, and also to the perspective of their application in different remediation technologies. Using enrichment procedures, I isolated two moderately halophilic Gram-negative bacterial strains from seawater sample, which are tolerant to organic solvents. Cell tolerance, adhesion and cells viability of *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} in the presence of organic solvents depends not only on its physicochemical properties and its concentration, but also on the specific response of the cells, and the cellular response is not the same for these bacterial strains. *n*-hexane, *n*-heptane, propylbenzene, with log P_{OW} between 3.69 and 4.39, were less toxic for *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3}, compared with toluene, styrene, xylene isomers and ethylbenzene, with log P_{OW} between 2.64 and 3.17. The results indicated that *Aeromonas salmonicida* IBB_{Ct2} is more susceptible to organic solvents than *Pseudomonas aeruginosa* IBB_{Ct3}. The mechanisms underlying solvent tolerance (e.g., the existance of the efflux pumps) in *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} it was also studied.

***Keywords*—**bacteria, mechanisms, organic solvent, resistance.

I. Introduction

EXTREMOPHILES are adapted to live under conditions of extreme temperature, pH, salinity, or pressure. Some organic solvents, as pollutants originating from human activities, also create extreme environmental conditions, and bacteria that are able to tolerate such environments have been recognized as a subgroup of the extremophiles [1]–[3]. The naturally produced solvents are present in low concentrations and they can be mineralized by microbial activities, and many of the metabolic pathways involved have been elucidated for various organisms and for a great number of compounds [4]–[5]. One of the major problems encountered in the application of these microbial mineralization processes in waste water and waste gas treatment or in bioremediation is the low stability of the desired activity as the result of inactivation of the cells, caused by the toxic effects of several pollutants [2],[6]–[11]. Among the most toxic chemicals are organic solvents, such as toluene, xylenes and styrene, which dissolve in the cell membrane, disorganize it, cause the loss of lipids and proteins, and eventually lead to cell death [2], [8]–[10], [12]–[15]. Microorganisms have developed various mechanisms to resist the lethal effects of organic solvents. Several physiological and biochemical approaches have been applied in an effort to elucidate the mechanisms of bacterial resistance to organic solvents. These studies have revealed a number of mechanisms, including: (1) metabolism of toxic compounds, which can contribute to their transformation into non-toxic compounds; (2) rigidification of the cell membrane via alteration of the phospholipid composition; (3) alteration in the cell surface that make the cells less permeable; (4) formation of vesicles that remove the solvent from the cell surface; and (5) efflux of the toxic compounds in an energy-dependent process [2], [9]–[13], [16]. One of the most relevant of these mechanisms is the active reduction of organic solvents entry into the cells through the action of membrane efflux pumps, which belong to the group of multidrug resistance (MDR) pumps. These efflux pumps extrude a broad range of structurally unrelated synthetic and natural chemicals and thus constitute an effective barrier against toxic chemicals [10], [15]. P-glycoprotein, a well characterized ABC-transporter of multidrug resistance family, bind a large number of lipophilic compounds, such as antibiotics, dyes, organic solvents and other toxic compounds, with no apparent structural or functional similarities and mediate the energy-dependent efflux of these toxic compounds from the bacterial cells [9], [17], [18].

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 [14], [15], [19], [20]. In this paper, I am presenting the isolation and characterization of two new moderately halophilic Gram-negative (*Aeromonas salmonicida* IBB_{Ct2}, *Pseudomonas aeruginosa* IBB_{Ct3}) bacteria that are tolerant to organic solvents (1% v/v *n*-hexane, *n*-heptane, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene). The toxic effects of these organic solvents on *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} bacterial strains, and the mechanisms behind their resistance, are also presented in this study.

II. Matherials and Methods

Isolation and characterization of solvent-tolerant bacterial strains. The number of viable bacterial strains in seawater sample (Constanta County, Romania) was estimated by a modified most probable number (MPN) procedure.

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Aliquots of 20 µl were added into ten separate dilution series in 96-microwell plates (Iwaki, London, United Kingdom). The wells were pre-filled with 180 µl saline (NaCl 2% w/v) minimal medium [14] and 10 µl sterilized crude oil or organic solvents and sealed. After 2 weeks incubation at 28°C, each well was added with 50 µl filter-sterilized solution of the respiration indicator 0.3% (w/v) INT [2-(4-iodophenyl)-3(4-nitrophenyl)-5-phenyltetrazolium chloride]. After over-night incubation in the dark at room temperature, red and pink wells were counted as positive for growth. A maximum-likelihood estimation of microbial numbers based on 10-fold dilution series was developed for the Microsoft Excel for Windows spreadsheet program [21], [22].

Isolation of IBB_{Ct2}, IBB_{Ct3} bacterial strains from seawater sample was carried out on saline (NaCl 2% w/v) minimal medium [14], using the enriched cultures method, with organic solvents (1% v/v *n*-hexane, *n*-heptane, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) as single carbon source. For further characterization of IBB_{Ct2} and IBB_{Ct3} solvent-tolerant bacterial strains several physiological and biochemical tests were performed: Gram reaction, growth on medium with NaCl, morphology, endospore formation, mobility, respirator type, pigments production, growth on Coomassie brilliant blue R250 agar (CBB agar), 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 IBB_{Ct2} and IBB_{Ct3} bacterial strains were determined based on their phenotypic characteristics and also based on the G+C content of the bacterial chromosome [23]. MICs of antimicrobial agents (e.g., penicillin, erythromycin, gentamycin, kanamycin, tetracycline, rhodamine 6G, bromophenol blue, sodium dodecyl sulfate) for IBB_{Ct2} and IBB_{Ct3} bacterial strains were also determined. Bacterial cells (30 µl, 10^7 CFU·ml^{-1}) were spotted on solid reach saline [14] medium (control) and on the same medium with antimicrobial agents at various concentrations (1-1000 µg·ml^{-1}). Petri dishes were incubated 24 hours at 28°C. MICs of antimicrobial agents were determined as the concentrations that severely inhibited bacterial cell growth. Here, as elsewhere in this work, experiments were repeated at least three times.

Solvent tolerance of bacterial strains. Bacterial cells (10^7 CFU·ml^{-1}) were cultivated on liquid or solid reach saline [14] medium (control) and on the same media in the presence of 0.1, 0.5 and 1% (v/v) organic solvents (*n*-hexane, *n*-heptane, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene). Flasks were sealed and incubated 24 hours at 28°C on a rotary shaker (150-200 rpm). Petri plates were also sealed and incubated 24 hours at 28°C.

1. ***Bacterial cells growth in the presence of organic solvents.*** Growth of the bacterial cells in liquid reach saline medium in the presence of different organic solvents was assessed by measuring turbidity (OD_{660nm}) after 24 hours. Growth on solid reach saline medium overlaid with organic solvents or supplied with organic solvents in the vapor phase was assessed as described by Nielsen *et al.* [3] and Ramos *et al.* [24], respectively.
2. ***Bacterial cells adhesion to organic solvents.*** Bacterial adhesion to organic solvents was determined using the method of Rosenberg *et al.* [25]. The bacterial adhesion to organic solvents was also studied on wet mount with the optical microscope.
3. ***Bacterial cells viability in the presence of organic solvents.*** Serial dilutions of culture liquid were spread on agar reach saline medium using the method of Ramos *et al.* [26] and the number of viable cells (CFU·ml^{-1}) was determined.

Membrane efflux pumps in solvent-tolerant bacterial strains. Bacterial cells (10^7 CFU·ml^{-1}) were cultivated on liquid reach saline medium (control) and on the same medium in the presence of 0.5% (v/v) *n*-heptane, toluene and ethylbenzene. Flasks were sealed and incubated 24 hours at 28°C on a rotary shaker (150-200 rpm).

1. ***Observation of protein profile modification in the presence of organic solvents.*** Membrane and periplasmic protein fractions were extracted with HE buffer (10 mM HEPES-NaOH, pH 7.6, 10 mM EDTA, 10 mM $MgCl_2$) solved in Laemmli buffer and denaturated at 95°C, for 5 min. 30 µg of protein per lane were loaded onto a 12% (w/v) polyacrylamide gel [27]. Gels were stained with Bio-Safe colloidal Coomassie Blue G-250 (Bio-Rad). Protein content was measured by the method of Bradford [28].
2. ***Observation of rhodamine 6G accumulation in the presence of organic solvents.*** Bacterial cells were spotted on solid saline medium (control) and on the same medium with 100 µg·ml^{-1} rhodamine 6G. Rhodamine 6G accumulation in bacterial cells was observed under UV light after 24 hours of incubation at 28°C.
3. ***Specific amplification of HAE1 (hydrophobe/amphiphile efflux 1) gene fragment by PCR***. Template DNA for PCR was obtained using the method of Whyte *et al.* [29]. 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 50 to 60°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 0.8% (w/v) TBE agarose gel and staining with fast blast DNA stain (Bio-Rad) the amplified fragments were analyzed.

Induced mutagenesis for solvent-tolerant bacterial strains. IBB_{Ct2} and IBB_{Ct3} bacterial strains were cultivated on liquid reach saline medium supplemented with 50 µg·ml^{-1} penicillin and gentamycin, respectively. Flasks were incubated 24 hours at 28°C on a rotary shaker (150-200 rpm). Recipient (IBB_{Ct2} bacterial strain) and donor (IBB_{Ct3} bacterial strain) cultures were washed twice with sterile liquid conjugation

medium [27] to eliminate any remains of antibiotic and salts. The pellets were suspended in 200 µl of the same medium, mixed at a ratio of 1:1 and placed onto a nitrocellulose membrane (pore size 0.22 µm, Millipore Co.) laid on the surface of the solid reach saline medium. The plates were incubated 20 hours at 28°C. Liquid reach saline medium supplemented with 10 mM sodium succinate and 50 $\mu g \cdot ml^{-1}$ gentamycin was used for selecting the mutants of IBB_{Ct2m} obtained after conjugation. Mutation frequency was determined as being the number of mutant cells expressing resistance to the gentamycin per total number of recipient cells. The bacterial cells were lysed with TE buffer (10 mM Tris-HCl, 1 mM $EDTANa_2$) 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. After separation on 0.8% (w/v) TBE (Tris-Borate-EDTA) agarose gel and ethidium bromide staining the DNA was visualized with ultraviolet. DNA content and purity were measured by the method of Sambrook *et al.* [27].

Reagents. *n*-hexane (96% pure), toluene (99% pure), styrene (99% pure), *o*-xylene (99% pure), *p*-xylene (99% pure), propylbenzene (98% pure) were obtained from Merck (E. Merck, Darmstadt, Germany), *n*-heptane (99% pure), *m*-xylene (98% 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).

III. Results and Discussion

Saline environments are often contaminated with organic solvents as a result of industrial activities and urban water effluents. Thus, the identification of moderate halophiles that play an important role in the degradation of toxic organic solvents is of great interest in the context of assessing acceleration of the environmental repair process for bioremediation in contaminated saline habitats [2], [10], [14], [15], [20]. Bacteria that live in extremes of salinity have made extensive modification of protein structures, lipid composition, ionic content of their cytoplasm, and metabolic pathways. Although these modifications have allowed such microorganisms to make efficient use of their particular environment, most of them have very limited abilities to make further adaptations to adjust to major changes in their environment [30].

Isolation and characterization of solvent-tolerant bacterial strains. Whereas no technique to enumerate specific metabolic types of bacteria in marine systems is absolute, the most probable number (MPN) technique can give consistent results that are appropriate for relative comparisons among sampling sites. Walker and Colwell [31] compared various methods for enumerating petroleum-degrading bacteria, and later Roubal and Atlas [32] developed the ^{14}C-labeled hydrocarbon-spiked crude oil MPN. Improved medium formulations for plate counts have been suggested, or, when oil is used as a sole carbon source, turbidity in microtiter plates [33] and oxygen consumption in test tubes [34] have been used for MPN determinations of hydrocarbon-degrading bacteria. More recently, a 96-well microtiter plate MPN procedure was developed to enumerate hydrocarbon-degrading bacteria [21]. Selective saline mineral medium with crude oil or organic solvents, as the sole carbon source, was used to enumerate the hydrocarbon-degrading bacteria in seawater sample (Constanta County, Romania) (Fig. 1). Growth in 96-microwell plates was identified using INT, which forms an insoluble red precipitate in wells containing bacteria that can use crude oil or organic solvents as a sole carbon source. After each plate from the 96-microwell plate had been scored, the number of positive wells at each serial dilution was entered into a computer program to determine the most probable number of bacteria per ml of seawater sample ($MPN \cdot ml^{-1}$ were between 10^2 and 10^{10}).

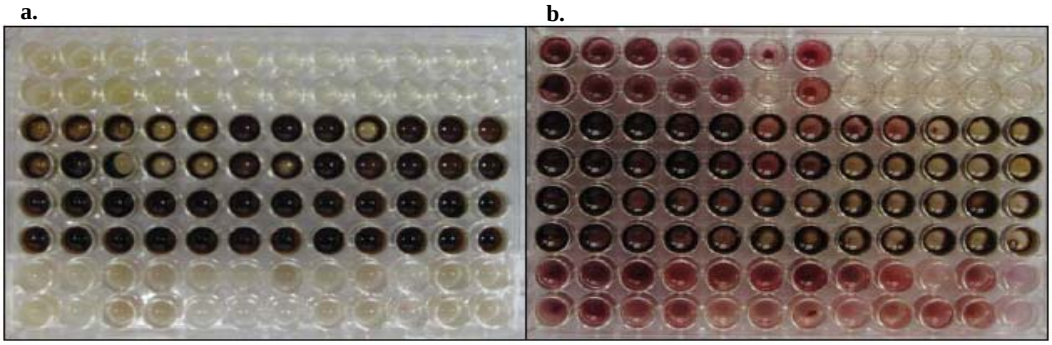


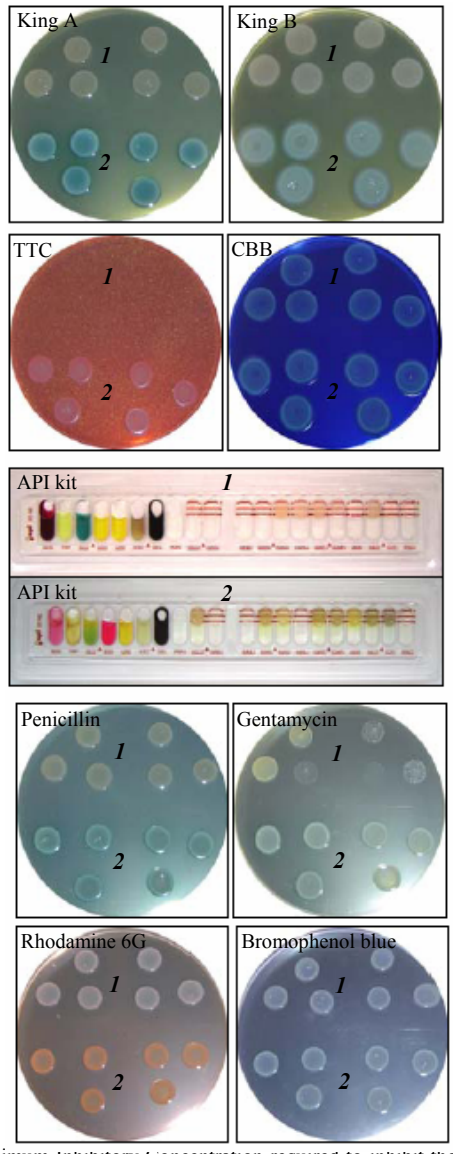
Fig. 1 MPN determination of hydrocarbon-degrading bacteria in 96-microwell plates

After 2 weeks incubation at 28°C (panel **a.**), each well was added with INT (panel **b.**) which forms an insoluble red precipitate in wells containing bacteria that can use crude oil or organic solvents as a sole carbon source.

Isolation of IBB_{Ct2} and IBB_{Ct3} bacterial strains from seawater sample was carried out on saline minimal medium, using the enriched cultures method, with organic solvents as single carbon source. The use of saline minimal medium with organic solvents (1% v/v *n*-hexane, *n*-heptane, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) as single carbon source allowed the selective development of IBB_{Ct2} and IBB_{Ct3} solvent-tolerant bacterial strains. The taxonomic affiliation of bacterial strains (*Aeromonas salmonicida* IBB_{Ct2}, *Pseudomonas aeruginosa* IBB_{Ct3}) was determined based on their phenotypic characteristics and also based on the G+C content of the bacterial chromosome (Table I). Both isolated solvent-tolerant bacteria belong to Gamma-*Proteobacteria*. Previous studies have reported that the dominance of Gamma-*Proteobacteria* is a characteristic of bacterial communities inhabiting environments contaminated with petroleum compounds and organic solvents [35], [36]. More than 20 genera of marine hydrocarbon-degrading bacteria, distributed over several (sub)phyla (Alpha-, Beta-, and Gamma-*Proteobacteria*) have been described so far [37]. The genome sequence divulges mechanisms contributing to metabolic fitness that allow these microorganisms to grow in a variety of ecosystems and begins to explain how *Aeromonas salmonicida* and *Pseudomonas aeruginosa* are able to survive in highly polluted environments. Thus, the versatility of these microorganisms, and also other bacteria described previously merits the sobriquet "jack of all trades" [38].

TABLE I PHENOTYPIC CHARACTERISTICS OF SOLVENT-TOLERANT BACTERIAL STRAINS

Characteristics		Strains	
		***A. salmonicida* IBB_{Ct2}**	***P. aeruginosa* IBB_{Ct3}**
Gram reaction		-	-
Optimal growth temperature		28°C	28°C
Growth on medium with NaCl		5%	10%
Morphology		rods	rods
Endospore formation		-	-
Mobility		+	+
Respirator type		A, FA	A
Pigment formation on:	LB	orange	green
	King A	-	+
	King B	-	-
Growth on TTC medium		-	+
Growth on CBB R_{250}		+	+
Catalase		+	+
Oxidase		+	+
Nitrates reduction		+	+
Indole production		-	-
D-glucose fermentation		-	-
L-arginine dihydrolase		-	+
Urease		-	+
Esculin hydrolysis		+	-
Gelatin hydrolysis		+	+
β-galactosidase		-	-
D-glucose assimilation		+	+
L-arabinose assimilation		-	-
D-mannose assimilation		-	-
D-mannitol assimilation		+	+
N-acetyl-glucosamine assimilation		+	-
D-maltose assimilation		+	-
Potassium gluconate assimilation		+	+
Capric acid assimilation		+	+
Adipic acid assimilation		-	+
Malic acid assimilation		+	+
Trisodium citrate assimilation		-	+
Phenylacetic acid assimilation		-	-
G+C content (%)		54.3	66.3
Phylogenetic branch		Gamma-*Proteobacteria*	Gamma-*Proteobacteria*
MIC_{90} (μg ml^{-1})	Penicillin	75	250
	Erythromycin	25	300
	Gentamycin	20	75
	Kanamycin	25	100
	Tetracycline	15	200
	Rhodamine 6G	250	500
	Bromophenol blue	50	100
	Sodium dodecyl sulfate	250	500



Legend: - = negative, + = positive, A, FA = aerobic, facultative anaerobic; A = aerobic; MIC_{90} = Minimum Inhibitory Concentration required to inhibit the growth of 90% of the bacteria. *Aeromonas salmonicida* IBB_{Ct2} (***1***) and *Pseudomonas aeruginosa* IBB_{Ct3} (***2***) bacterial strains.

The resistance of *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} to antimicrobial agents (such as penicillin, erythromycin, gentamycin, kanamycin, tetracycline, rhodamine 6G, bromophenol blue, sodium dodecyl sulfate) differs from one strain to another (Table I). Antimicrobial effect of all these compounds was more pronounced for *Aeromonas salmonicida* IBB_{Ct2} (MIC_{90} = 15 - 250 μg·ml^{-1}), compared with antimicrobial effect of the some compounds for *Pseudomonas aeruginosa* IBB_{Ct3} (MIC_{90} = 75 - 500 μg ml^{-1}). In natural environments, microorganisms are exposed to changing conditions and have therefore developed a series of strategies to cope with these stressors [15]. According with literature [2], the survival of the well-studied *Pseudomonas* strains in the presence of different antimicrobial compounds in high concentration is based on their ability to induce or activate a broad range of different adaptation mechanisms. Many of these mechanisms can also be found in nontolerant bacteria, or they are known as a defense to other antimicrobial compounds. The increasing antibiotic resistance in bacteria can be caused by diverse mechanisms involving decreased antibiotic accumulation, physical modification or destruction of the antibiotics, and alteration of the enzyme target of these agents. In recent years, a mechanism of resistance involving the active efflux of antibiotics by pumps has been elucidated. These efflux systems are broadly specific and able to accommodate a variety of structurally unrelated antimicrobial agents, such as antibiotics, dyes, detergents, biocides, fatty acids, and organic solvents [10], [13], [14], [39]–[41].

Solvent tolerance of bacterial strains. Organic solvents have many detrimental effects on bacteria, and some solvents are more harmful than others. To classify the intrinsic toxicity of a solvent, the logarithm of its partition coefficient in *n*-octanol and water was measured and termed log P_{OW}. Solvents with low log P_{OW} (1.5 to 3.5) are considered extremely toxic, while solvents with higher log P_{OW} are less toxic. The reasoning for this toxicity index lies in the fact that solvents with lower log P_{OW} values tend to partition into the cytoplasmic membranes of organisms, compromising the structure and destroying vital functions [3], [9], [10], [12]. In recent years, a growing number of bacterial species able to overcome these toxic effects have been found. Most solvent-tolerant bacterial species are Gram-negative. Gram-negative bacteria have the advantage of having an additional outer membrane, which allows quick modifications and adaptations in the lipopolysaccharides, efflux pumps, and/or fatty acid composition [2], [3], [14], [26], [42].

Bacterial cells growth in the presence of organic solvents depends not only on the inherent toxicity of the solvent, but also on the intrinsic tolerance of the bacterial strain. Strains of *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} exhibited exceptional organic solvents tolerance (v/v) (Table II).

TABLE II SOLVENT TOLERANCE OF BACTERIAL STRAINS

Variant	log P_{OW} [a]	Solvent conc. (v/v)	Growth in the presence of organic solvents (%) [b]		Adhesion to organic solvents (%) [c]		Cells viability in the presence of organic solvents (CFU·ml^{-1}) [d]	
			A. salmonicida IBB_{Ct2}	*P. aeruginosa* IBB_{Ct3}	*A. salmonicida* IBB_{Ct2}	*P. aeruginosa* IBB_{Ct3}	*A. salmonicida* IBB_{Ct2}	*P. aeruginosa* IBB_{Ct3}
Control	-		100	100	-	-	$3{,}1\times10^{9}$	$3{,}2\times10^{9}$
		0.1%	100	100	45.4	60.0	3.0×10^{9}	3.0×10^{9}
n-Hexane	3.86	0.5%	75	100	40.2	50.2	3.3×10^{8}	2.0×10^{9}
		1.0%	50	75	23.9	34.7	3.7×10^{5}	4.9×10^{8}
		0.1%	100	100	49.7	61.4	4.3×10^{8}	2.3×10^{9}
n-Heptane	4.39	0.5%	75	100	35.8	55.3	1.4×10^{8}	1.3×10^{9}
		1.0%	50	100	27.9	44.6	4.0×10^{6}	9.8×10^{8}
		0.1%	50	75	14.6	29.2	3.6×10^{3}	2.3×10^{8}
Toluene	2.64	0.5%	25	50	7.2	13.8	2.5×10^{3}	2.3×10^{7}
		1.0%	0	25	0.3	5.6	0	1.0×10
		0.1%	50	75	5.6	10.6	1.2×10^{3}	1.7×10^{3}
Styrene	2.86	0.5%	25	50	0.8	6.8	1.8×10^{2}	1.0×10^{2}
		1.0%	0	25	0.2	3.9	0	1.3×10
	3.09	0.1%	100	100	23.3	30.5	5.0×10^{4}	8.7×10^{7}
o-Xylene		0.5%	50	75	16.7	24.6	2.0×10^{3}	1.7×10^{6}
		1.0%	25	75	9.7	15.1	0	8.7×10^{3}
	3.14	0.1%	100	100	26.6	35.4	1.0×10^{5}	6.0×10^{8}
m-Xylene		0.5%	75	100	17.8	28.8	4.4×10^{4}	1.0×10^{6}
		1.0%	50	75	16.9	20.2	5.3×10^{2}	1.0×10^{5}
		0.1%	75	100	20.5	33.2	1.0×10^{2}	3.1×10^{7}
p-Xylene	3.14	0.5%	50	75	14.8	26.6	1.5×10	2.4×10^{5}
		1.0%	25	50	8.9	17.4	0	4.6×10^{3}
		0.1%	100	100	10.2	13.8	1.5×10^{4}	1.5×10^{5}
Ethylbenzene	3.17	0.5%	50	75	6.9	12.4	5.6×10^{3}	6.7×10^{4}
		1.0%	25	50	2.3	7.8	5.6×10	6.7×10^{3}
		0.1%	100	100	23.6	31.6	8.7×10^{5}	8.7×10^{6}
Propylbenzene	3.69	0.5%	75	100	14.9	27.2	3.5×10^{4}	8.2×10^{5}
		1.0%	50	75	7.6	13.2	3.0×10^{3}	4.5×10^{3}

Legend: [a] = logarithm of the partition coefficient of the solvents in octanol-water mixture; [b] = the growth on liquid reach saline medium in the presence of different organic solvents was estimated by measuring turbidity, and the tolerance is represented by the frequency of growth, with that observed in the absence of any organic solvent taken as 100%; [c] = the decrease of the turbidity in aqueous phase in the presence of organic solvents (% BATS); [d] = serial dilutions of culture liquid were spread on agar medium and the number of viable cells (CFU·ml^{-1}) was determined; *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} cell growth, adhesion and cells viability in the presence of organic solvents were determined after 24 hours incubation in the presence of 0.1-1% (v/v) organic solvents.

n-hexane, *n*-heptane, propylbenzene, with log P_{OW} between 3.69 and 4.39, were less toxic (50-100%) for these bacterial strains, compared with toluene, styrene, xylene isomers and ethylbenzene (0-100%), with log P_{OW} between 2.64 and 3.17. *Aeromonas salmonicida* IBB_{Ct2} was more susceptible (0-100%) to organic solvents, compared with *Pseudomonas aeruginosa* IBB_{Ct3} (25-100%).

The growth of the bacterial strains on agar reach saline medium overlaid with organic solvents or with organic solvents in vapor phase was 0-50% for *Aeromonas salmonicida* IBB_{Ct2}, and 0-100% for *Pseudomonas aeruginosa* IBB_{Ct3} (Fig. 2). In plate overlay assays (30 µl, 10^7 CFU·ml^{-1}) *Aeromonas salmonicida* IBB_{Ct2} tolerated only *n*-heptane, while *Pseudomonas aeruginosa* IBB_{Ct3} tolerated, for 6 hours, *n*-hexane, *n*-heptane, styrene, xylene isomers, ethylbenzene, and propylbenzene. After removal of the organic solvents and subsequent incubation at 28°C, confluent cell growth was seen after 24 hours (Fig. 2a). In plate vapor phase assays (30 µl, 10^7 CFU·ml^{-1}) *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} tolerated all organic solvents (except toluene) and confluent cell growth was seen after 24 hours (Fig. 2b). Although these bacterial strains were able to use 0.1-1% organic solvents in liquid reach saline medium (except 1% v/v toluene and styrene), they were not able to grow, in some cares, on agar reach saline medium with organic solvents in the vapor phase or overlaid with organic solvents. This can be explained by the fragmentation of liquid medium surface film in small droplets, more available to bacterial degradation, due to the stirring (150-200 rpm).

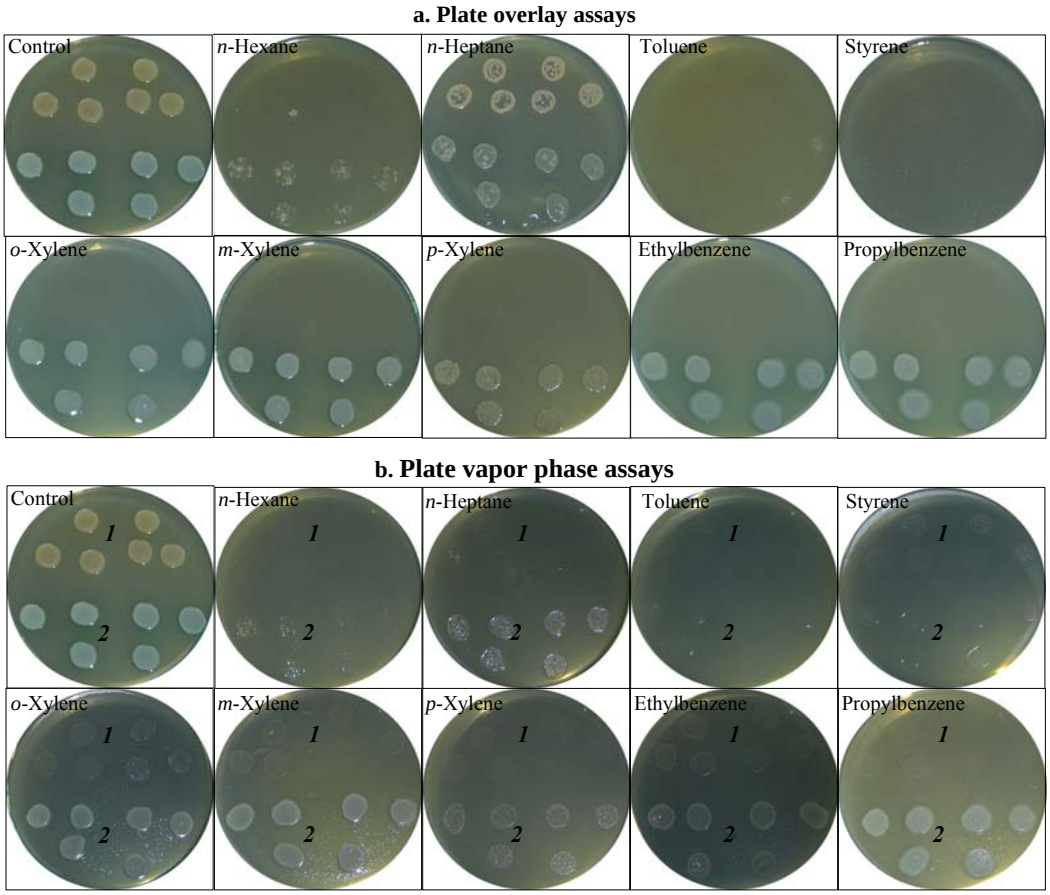


Fig. 2 Solvent tolerance of bacterial strains

Growth of *Aeromonas salmonicida* IBB_{Ct2} (spots ***1***) and *Pseudomonas aeruginosa* IBB_{Ct3} (spots ***2***) on agar reach saline medium overlaid with organic solvents (panel **a.**) or with organic solvents in vapor phase (panel **b.**) was estimated by determining the formation of resistant bacterial colonies, and the tolerance is represented by the frequency of growth, with that observed in the absence of any organic solvent taken as 100%.

Bacterial cells adhesion to organic solvents assay measures the partitioning of cells between aqueous and hydrophobic phases. It should be emphasized that, whereas this partitioning is related to cell surface hydrophobicity, bacterial adhesion assay results do not represent an absolute value of cell surface hydrophobicity. Rather, bacterial adhesion assay results are relative and can be used to compare the response of cells grown under various conditions [43]. The outer membrane was shown to be engaged in promoting solvent tolerance. Ions such as Mg^{2+} or Ca^{2+} stabilize the organization of the outer membrane and contribute to a higher resistance of solvent-tolerant *Pseudomonas* strains toward toluene [10], [44]. After adaptation to toluene, solvent-tolerant *Pseudomonas putida* S12 cells become less hydrophobic [45]. Recently, it was shown that a reduction of the cell hydrophobicity correlates with changes in the lipopolysaccharide (LPS) content [1], [10].

In this assay the bacterial cells adhered to organic solvents (0.1-1% v/v) microdroplets formed as a result of mechanical dispersion, which are stable, causing the decrease of the turbidity in the aqueous phase. Both, *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} cells presented higher (7.6-61.4%) hydrophobicity when the growth was done on reach saline medium in the presence of *n*-hexane, *n*-heptane and propylbenzene, compared with cell hydrophobicity (0.2-33.2%) when the growth was done on the same medium but in the presence of toluene, styrene, xylene isomers and ethylbenzene (Table II), fact confirmed by the optical microscope observations (data not shown). Also, I observed a decrease in hydrophobicity with the increase of organic solvents concentration in culture medium. 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 [1], [2], [9], [10].

Bacterial cells viability in the presence of organic solvents. Against the idea that the toxicity of a second phase of a organic solvent can be inferred from its hydrophobicity, reflected by its log P_{OW}, it was observed that some marine toluene-tolerant bacteria were unable to grow in the presence of some solvents with log P_{OW} values higher than toluene. To explain these findings, the different partition of the solvent in the membrane was invoked. However, the toxicity of a given organic solvent depends not only on its physicochemical properties but also on the specific response of the cells, and that this cellular response is not the same in all strains [14].

The viability of *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} cells to organic solvents differ from one strain to another and even for the same bacterial strain, according to the nature of hydrophobic substrate and its concentration (Table II). The bacterial cells presented a higher viability ($CFU \cdot ml^{-1} = 10^3$-10^9) when growth was done on reach saline medium, in the presence of *n*-hexane, *n*-heptane and propylbenzene, compared with the viability of the bacterial cells ($CFU \cdot ml^{-1} = 0$-10^8) grown in the presence of toluene, styrene, xylene isomers and ethylbenzene. *n*-hexane, *n*-heptane, propylbenzene, with log P_{OW} between 3.69 and 4.39, were binding less abundantly to viable bacterial cells, being less toxic for them, while toluene, styrene, xylene isomers and ethylbenzene with log P_{OW} between 2.64 and 3.17 were binding more abundantly to viable bacterial cells, being more toxic for them. I observed a decrease in cell viability with the increase of organic solvents concentration (0.1-1% v/v) in culture medium. The survival rates of *Aeromonas salmonicida* IBB_{Ct2} cells decreased significantly, below the detection limit of the experiment, in the presence of 1% (v/v) toluene, styrene, *o*-xylene and *p*-xylene.

Membrane efflux pumps in solvent-tolerant bacterial strains. The mechanisms underlying organic solvent tolerance are not yet fully understood, but a number of factors involved in the process have been characterized over the last ten years. Several laboratories have identified constitutive and inducible efflux pumps belonging to the RND (resistance, nodulation, and cell division) family as being involved in organic solvent tolerance [3], [10]–[12], [46], [47]. These bacterial efflux pumps are made of three components: an efflux pump transporter located in the cytoplasmic membrane that recognizes substrates in the periplasm or in the outer leaflet of the cytoplasmic membrane, an outer membrane protein which forms a trimeric channel that penetrates into the periplasm and contacts the efflux pump transporter, and a lipoprotein anchored to the inner membrane which expands into the periplasmic space and may serve as a bracket for the other two components [48].

Observation of protein profile modification in the presence of organic solvents. Organic solvents are known to be toxic for the cells, mainly due to their effect on the membranes as so-called "membrane-active compounds" [9], [11], [49]. To investigate the modifications induced by organic solvents to membrane and periplasmic protein profile of *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} cells, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis was perform. The electrophoresis studies showed the existence of some differences between protein patterns extracted from bacterial cells incubated without organic solvents (control) and those extracted from bacterial cells incubated 24 hours in the presence of 0.5% (v/v) *n*-heptane, toluene and ethylbenzene (Fig. 3a). Modifications induced were different from one strain to another. I observed induction of the synthesis of some proteins in the membrane and periplasmic protein profile of *Aeromonas salmonicida* IBB_{Ct2} cells grown in the presence of toluene and ethylbenzene, compared with the control and no modifications were observed in the presence of *n*-heptane. I observed strong induction of the synthesis of some proteins in the membrane and periplasmic profile of the *Pseudomonas aeruginosa* IBB_{Ct3} cells grown in the presence of *n*-heptane, toluene and ethylbenzene, compared with the control. Recently, an extended proteomic survey was performed to identify all processes responsible for *Pseudomonas putida* DOT-T1E adaptation to toluene, showing that a whole cascade of mechanisms is necessary to allow the bacterium to survive in the presence of such toxic compound. The presence of toluene in the culture medium in which *Pseudomonas putida* DOT-T1E has been cultured provoked drastic changes in the protein pattern. As part of this response, new proteins involved in solvent tolerance were synthesized and an increased expression of some preexisting proteins also took place, counteracting the decrease in activity due to membrane structural damage caused by the presence of toluene inside the cell [13].

Observation of rhodamine 6G accumulation in the presence of organic solvents. The existence of solvent efflux pumps in *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} cells was confirmed by rhodamine 6G accumulations ($100\ \mu g \cdot ml^{-1}$) in bacterial cells (Fig. 3b). Accumulation of rhodamine 6G in bacterial cells was observed by the fluorescence of rhodamine 6G under UV light. According the literature [18], rhodamine 6G is P-glycoproteins substrates, which mediate the energy-dependent efflux of certain toxic compounds from the bacterial cells. The rhodamine 6G accumulation assays showed the existence of some differences between bacterial cells incubated without

organic solvents (control) and those incubated 24 hours in the presence of 0.5% (v/v) *n*-heptane, toluene and ethylbenzene. I observed an increase of rhodamine 6G accumulation in *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} cells grown in the presence of toluene and ethylbenzene, compared with the control. No significant modifications in rhodamine 6G accumulation were observed in *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} cells grown in the presence of *n*-heptane, compared with the control.

Specific amplification of HAE1 (hydrophobe/amphiphile efflux 1) gene fragment by PCR. To determine whether efflux pumps of RND family were present in *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} (Fig. 4a), it was performed an amplification reaction with oligonucleotides A24f2 and A577r2 [50].

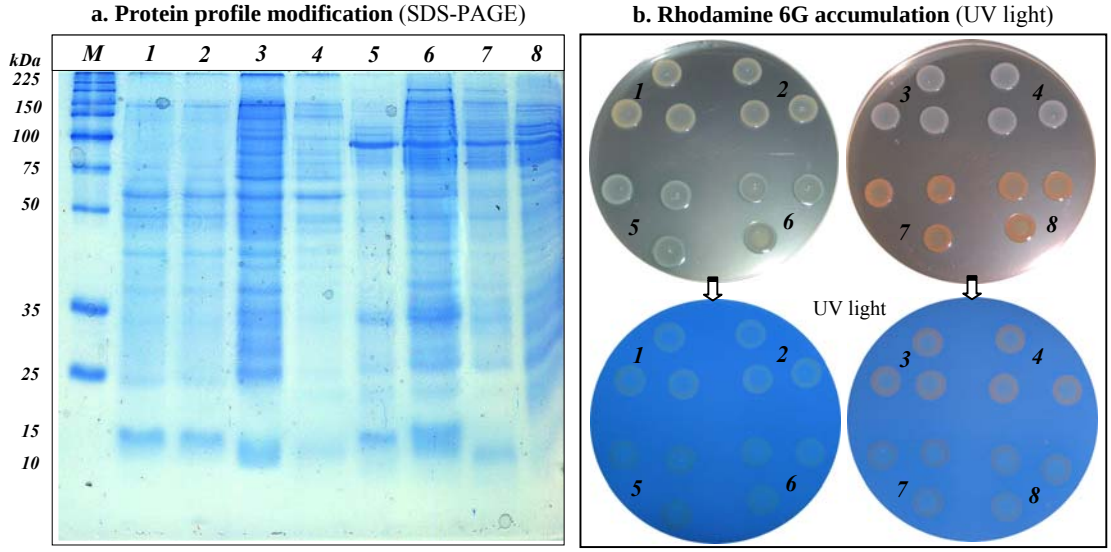


Fig. 3 Membrane efflux pumps in solvent-tolerant bacterial strains

The protein profile modification (panel **a.**) and rhodamine 6G accumulation (panel **b.**) in *Aeromonas salmonicida* IBB_{Ct2} (lanes or spots ***1-4***) and *Pseudomonas aeruginosa* IBB_{Ct3} (lanes or spots ***5-8***); bacterial strains cultivated onto liquid reach saline medium without organic solvents (lanes or spots ***1***, ***5***); bacterial strains cultivated onto liquid reach saline medium with 0.5% (v/v) *n*-heptane (lanes or spots ***2***, ***6***), toluene (lanes or spots ***3***, ***7***) and ethylbenzene (lanes or spots ***4***, ***8***); broad range protein molecular weight marker, Promega (lane ***M***).

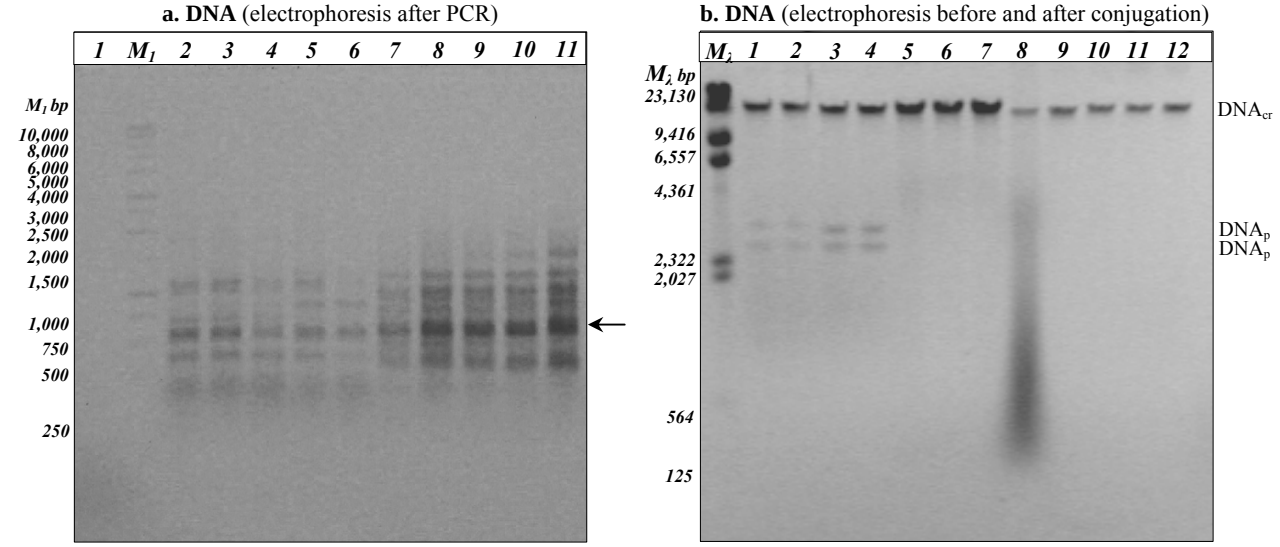


Fig. 4 PCR products of solvent-tolerant bacterial strains using primers that amplify HAE1 fragment (panel **a.**) and induced mutagenesis for solvent-tolerant bacterial strains (panel **b.**)

Panel **a.** Negative control DNA (lane ***1***); 1 kb DNA ladder, Promega (lane M_1); *Aeromonas salmonicida* IBB_{Ct2} (lanes ***2-6***); *Pseudomonas aeruginosa* IBB_{Ct3} (lanes ***7-11***); anneling at: 50.8°C (lanes ***2*** and ***7***), 52.4°C (lanes ***3*** and ***8***), 54.7°C (lanes ***4*** and ***9***), 57.6°C (lanes ***5*** and ***10***), 60.0°C (lanes ***6*** and ***11***); the positions of the expected HAE1 fragment are indicated with arrow. Panel **b.** Lambda DNA/*Hind* III Marker, Promega (lane ***M***); *Pseudomonas aeruginosa* Ct_3 (lanes ***1-4***), *Aeromonas salmonicida* Ct_2 (lanes ***5-8***) before conjugation; *Aeromonas salmonicida* IBB_{Ct2m} (lanes ***8-12***) after conjugation.

These primers were specifically designed to amplify the HAE1 family of transporters, which includes all the known drug- or solvent-resistant RND transporters plus 60 hypothetical transporters [14]. Both *Aeromonas salmonicida* IBB_{Ct2} and *Pseudomonas aeruginosa* IBB_{Ct3} amplified the expected 550-bp fragment and the optimum temperatures for primer annealing were 52.4°C (Fig. 4a).

Aeromonas salmonicida IBB_{Ct2} was more susceptible to organic solvents, compared with *Pseudomonas aeruginosa* IBB_{Ct3}. It is obvious that only a combination of mechanisms allows the survival of the solvent-tolerant strains. The regulation of such diverse response system may be connected to a general stress response. This is likely, as the tolerance to organic solvents correlates to the resistance toward other harmful environmental factors in the solvent-tolerant strains [2].

Induced mutagenesis for solvent-tolerant bacterial strains. Exposure of natural microbial populations to different organic solvents may impose a selective advantage to strains possessing plasmids encoding enzymes for solvent catabolism, resulting in an overall increase in the plasmid frequency in the community. Plasmid DNA play a particularly important role in genetic adaptation in that it represents a highly mobile form of DNA which can be transferred via conjugation or transformation and can impart novel phenotypes, including solvent degradation ability, to recipient organisms [51]. Conjugative plasmids are thus important agents of genetic changes and evolution in bacteria, and could be picked up from or brought together in different organisms as groups of genes, which through mutations and recombination can specify new metabolic functions [52]. Llamas *et al.* [53] considered that there are very few genetic studies that have been carried out on halophilic microorganisms, probably due to various difficulties related to the high salt concentrations that these microorganisms need to grow in. For example, some antibiotics used as markers in genetics are inactivated by salts and salinity and also acts negatively upon plasmid DNA transference. The exopolysaccharides produced by different bacterial strains represents an additional obstacle, because EPS behave as a barrier that interferes with induced mutagenesis [53], [54].

There was observed, by agarose gel electrophoresis studies (Fig. 4b), the existence of some plasmids in *Pseudomonas aeruginosa* IBB_{Ct3}, while *Aeromonas salmonicida* IBB_{Ct2} strain do not harbor plasmid. According with the literature, most of solvent-tolerant bacteria do not harbor plasmids, indicating that the resistance factors reside on the chromosome [16].

Initially, I looked into several factors in order to determine the optimum conditions (time and composition of culture media for mating, antibiotic susceptibility) for mating between *Aeromonas salmonicida* IBB_{Ct2} (recipient) and *Pseudomonas aeruginosa* IBB_{Ct3} (donor). Liquid reach saline medium supplemented with 10 mM sodium succinate and 50 $\mu g \cdot ml^{-1}$ gentamycin was used for selecting the mutants of *Aeromonas salmonicida* IBB_{Ct2m} obtained after conjugation. From independently isolated gentamycin-resistant mutants of *Aeromonas salmonicida* IBB_{Ct2m}, I extracted total DNA and it was observed that the plasmid did not exist inside of these bacterial cells (Fig. 4b). The increased resistance to gentamycin (from 20 to 50 $\mu g \cdot ml^{-1}$) of *Aeromonas salmonicida* IBB_{Ct2m} cells could be the result of the spontaneous mutagenesis. I also tested the tolerance of *Aeromonas salmonicida* IBB_{Ct2m} to organic solvents (*n*-hexane, *n*-heptane, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene), compared with the wild-type. The tolerance to organic solvents of *Aeromonas salmonicida* IBB_{Ct2m} (25-100%), was higher, compared with wild-type *Aeromonas salmonicida* IBB_{Ct2} (0-100%), data not shown. Further studies will be carried out on this topic, as well as the genomic DNA will be screened by PCR for the presence of catabolic genes involved in known solvent biodegradative pathways, and for the presence of other efflux pumps of the RND family.

IV. Conclusion

Although organic solvents are highly toxic for living organisms because they accumulate in and disrupt cell membranes, more and more bacterial strains have been obtained that can adapt to and survive these compounds. Initially, most solvent-tolerant strains isolated belong to the genus *Pseudomonas*. In the meantime, however, other genera have also been shown to include solvent-tolerant strains. The high world interest given to the researches concerning the study of solvent-tolerant bacteria is due to their high biotechnological potential, and also to the perspective of their application in different remediation technologies for polluted ecosystems, based on the use of solvent-tolerant bacteria isolated from polluted environments. The biotechnological importance of these bacteria makes them ideal candidates for genetic studies. Hence, studying solvent-tolerant bacteria and, particularly, the molecular mechanisms enabling them to survive such hostile environmental conditions will contribute to further developing efficient two-phase biotransformation systems with whole cells but will also provide deep new insights into the general stress response of bacteria.

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