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Research Paper

Response of *Rhodococcus erythropolis* strain IBB_{Po1} to toxic organic solvents

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Abstract

Recently, there has been a lot of interest in the utilization of rhodococci in the bioremediation of petroleum contaminated environments. This study investigates the response of *Rhodococcus erythropolis* IBB_{Po1} cells to 1% organic solvents (alkanes, aromatics). A combination of microbiology, biochemical, and molecular approaches were used to examine cell adaptation mechanisms likely to be pursued by this strain after 1% organic solvent exposure. *R. erythropolis* IBB_{Po1} was found to utilize 1% alkanes (cyclohexane, n-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) as the sole carbon source. Modifications in cell viability, cell morphology, membrane permeability, lipid profile, carotenoid pigments profile and 16S rRNA gene were revealed in *R. erythropolis* IBB_{Po1} cells grown 1 and 24 h on minimal medium in the presence of 1% alkanes (cyclohexane, n-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene). Due to its environmental origin and its metabolic potential, *R. erythropolis* IBB_{Po1} is an excellent candidate for the bioremediation of soils contaminated with crude oils and other toxic compounds. Moreover, the carotenoid pigments produced by this nonpathogenic Gram-positive bacterium have a variety of other potential applications.

Key words: *Rhodococcus*, solvents, morphology, lipids, carotenoids.

Introduction

The existence of contaminated areas with highly toxic organic solvents is a clear indication of the lack of biological systems that have the ability to efficiently degrade these compounds. Research in recent years has focused on the search for solvent-tolerant bacteria that have the catabolic potential necessary to remove comprehensively these toxic compounds (Torres *et al.*, 2011). Many of the organic solvents are relatively stable and toxic to bacteria because they are able to bind to the cell membrane (Torres *et al.*, 2011). When organic solvents accumulate in the cell membrane, its integrity is affected, resulting in loss of function as a permeability barrier, as a protein and reaction matrix and as an energy transducer leading concomitantly to damages of the cellular metabolism, growth inhibition, and, finally leading to cell death (Isken and de Bont, 1998; Heipieper *et al.*, 2007). The large genomes of *Rhodococcus* strains, their redundant and versatile catabolic pathways, their ability to uptake and metabolize hydrophobic compounds, to form biofilms, to persist in adverse conditions, as well as the availability of recently developed tools for genetic engineering in rhodococci make them suitable industrial microorganisms for biotransformations and the biodegradation of many toxic compounds (Larkin *et al.*, 2006; Martínková *et al.*, 2009). Furthermore, these bacteria are capable to produce several carotenoid pigments (*e.g.*, β-carotene, γ-carotene, chlorobactene) with different medical, industrial, and nutritional applications (Tao *et al.*, 2006). Some organic solvent tolerance mechanisms in rhodococci have been proposed (*e.g.*, induction of general stress regulon, production of organic solvent emulsifying or deactivating enzymes, active solvent efflux pumps) (Torres *et al.*, 2011). In response to toxic organic solvents cell morphology alterations and filamentous growth were also observed in some solvent-tolerant bacteria (Torres *et al.*, 2011). The variety of mechanisms that could confer adaptation to organic solvents implies that bacterial solvent tolerance cannot be provided by a single mechanism (Heipieper *et al.*, 2007).

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Taking into account the interest raised by the utilization of rhodococci in the bioremediation of petroleum contaminated environments, this study investigates the response of *Rhodococcus erythropolis* IBB_{Po1} cells to 1% organic solvents (alkanes, aromatics). Cyclohexane, *n*-hexane, *n*-decane, toluene, styrene, and ethylbenzene with different log P_{OW} values (logarithm of the partition coefficient in octanol-water mixture) have been used as the sole carbon source in minimal medium. The possible cell adaptation mechanisms pursued by this strain after 1% organic solvent (alkanes, aromatics) exposure was studied by following the changes in the cell viability, cell morphology, membrane permeability, lipid profile, carotenoid pigments profile, and 16S rRNA gene.

Materials and Methods

Bacterial strain

The strain used in the present study was *R. erythropolis* IBB_{Po1} (KF059972.1), which has been previously isolated in our laboratory from Poeni crude oil-contaminated soil sample (Stancu, 2014). The analysis of the 16S rRNA gene sequence located strain IBB_{Po1} (KF059972.1) within the genus *Rhodococcus*, showing 95% similarity to other *Rhodococcus* strains from the public databases (GenBank/DDBJ/EMBL).

Response of *R. erythropolis* IBB_{Po1} cells to organic solvents

Bacterial cells were cultivated on liquid LB medium containing 30 μg mL^{-1} kanamycin and incubated at 28 °C on a rotary shaker (200 rpm) until they reached the exponential growth phase. Bacterial cells were harvested by centrifugation, washed twice and finally resuspended (10^6 cells mL^{-1}) in minimal medium (Stancu and Grifoll, 2011). 0.1% (w/v) yeast extract or 1% (v/v) organic solvents (alkanes: cyclohexane, *n*-hexane, *n*-decane; aromatics: toluene, styrene, ethylbenzene) were supplied to the cell suspensions as the sole carbon source. Flasks were sealed and incubated for 1 and 24 h respectively at 28 °C on a rotary shaker (200 rpm).

Cell viability

Serial dilutions of bacterial cultures were spotted on LB agar and the number of viable cells (cfu mL^{-1}) was determined, using the method described by Segura *et al.* (2008). Petri plates were incubated 24 h at 28 °C.

Cell morphology

Cell morphology was studied using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM samples were prepared using the method indicated by Rocha *et al.* (2011). Samples were fixed on SEM holder and gold-coated with a JEOL JFC-1300 auto fine coater, in a deep vacuum. The samples were examined with a JEOL JSM-6610LV SEM. TEM samples were prepared using the method indicated by Gutiérrez *et al.* (2009). Thin sections were mounted onto 300-mesh collodion/carbon coated cooper grids and stained with lead citrate and uranyl acetate. Examination of the sectioned material was performed using a JEOL JEM-1400 80 kV TEM.

Membrane permeability

Membrane permeability was determined using the method indicated by Gaur and Khare (2009). To determine the release of nucleic acid from bacterial cells, the absorbance of cell-free supernatant was measured at 260 nm.

Membrane lipids

Membrane lipids were analyzed by thin layer chromatography (TLC). Lipids were extracted from the culture broths with chloroform-methanol (2:1 v/v). The samples were spotted with a Linomat 5 sample applicator (CAMAG), on a 10 x 20 cm precoated silica gel 60 TLC aluminium sheets (Merck). The separation was performed using chloroform-methanol-acetic acid-water (85:22.5:10:4 v/v/v/v) mixture as mobile phase (Das *et al.*, 2009). For phospholipids visualization, the plates were treated with 10% (w/v) molybdatophosphoric acid hydrate in ethanol, and for glycolipids, the plates were treated with 0.5% (w/v) α-naphthol in methanol-water (1:1 v/v) mixture and sulfuric acid-ethanol (1:1 v/v) mixture. The densitometric scan of dried TLC plates was performed at 500 nm with a TLC Scanner 4 (CAMAG).

Carotenoid pigments

Carotenoid pigments were analyzed by spectrophotometry and thin layer chromatography (TLC). Carotenoids were extracted from the culture broths with acetone (Tao *et al.*, 2006). UV/visible scanning spectra of the samples were recorded between 200 and 800 nm using a SPECORD 200 UV-visible spectrophotometer (Analytik Jena). For TLC analysis, the samples were spotted with a Linomat 5 sample applicator (CAMAG), on a 10 x 20 cm precoated silica gel 60 TLC aluminium sheets (Merck). The separation was performed using chloroform-methanol (90:10 v/v) mixture as mobile phase. The densitometric scan of dried TLC plates was performed at 254 nm with a TLC Scanner 4 (CAMAG).

16S rRNA gene expression

Genomic DNA was extracted using the method of Whyte *et al.* (1996). For PCR amplification of 16S rRNA gene, 2 μL of DNA extract was added to a final volume of 50 μL reaction mixture, containing: 5 x GoTaq flexi buffer, $MgCl_2$, dNTP mix, bacterial universal primers (27f and 1492r, Marchesi *et al.*, 1998), and GoTaq DNA polymerase

(Promega). PCR was performed with a mastercycler proS (Eppendorf). The PCR program consisted in initial denaturation for 10 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, extension step at 72 °C for 2 min, and a final extension at 72 °C for 10 min. After separation on 1.5% (w/v) TBE agarose gel (Sambrook *et al.*, 1989) and staining with fast blast DNA stain (Bio-Rad) the PCR products were analyzed. The PCR products were purified using the DNA clean and concentrator-5 kit (Zymo Research). PCR products were digested with *Eco*RI and *Xba*I restriction endonucleases (Promega), and the resultant restriction fragments were analyzed by electrophoresis on 2% (w/v) agarose gel. PCR products were also sequenced with the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems). The reactions were performed using the amplification primers. The products were purified using the BigDye XTerminator purification kit (Applied Biosystems). Sequencing reactions were obtained with an Applied Biosystems 3500/3500xL genetic analyzer at the Institute of Biology Bucharest of Romanian Academy. DNA sequencing runs were assembled using the BioEdit software (Hall, 1999). The sequences obtained were compared with respective control using the BLAST program (Altschul *et al.*, 1990). The phylogenetic tree was generated using the neighbor-joining methods in MEGA5.1 program (Tamura *et al.*, 2011).

Reagents used during this study were of reagent grade and purchased from different commercial sources (Merck, Sigma-Aldrich, Promega, Invitrogen, Zymo Research, Applied Biosystems, Bio-Rad Laboratories). The PCR primers were purchased from Integrated DNA Technologies.

Results and Discussion

Response of *R. erythropolis* IBB_{Po1} cells to organic solvents

Numerous and complex physiological cellular responses and adaptations involved in organic solvents tolerance have been observed in *R. erythropolis* IBB_{Po1} cells grown on LB medium in the presence of 1% organic solvents (Stancu, 2014). This bacterium was adapt to 1% organic solvents by changing surface hydrophobicity, cell morphology, metabolic fingerprinting and the *otsA1* (trehalose-6-phosphate synthase) gene sequence. Taking into account the interest raised by the utilization of rhodococci in the bioremediation of petroleum contaminated environments, we continued our previous study with the investigation of the response of *R. erythropolis* IBB_{Po1} cells to 1% organic solvents (alkanes, aromatics). In this study, 1% alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) were used as the sole carbon source in minimal medium. The controls were prepared using the same mineral medium, but supplemented with 0.1% (w/v) yeast extract as the carbon source.

Cell viability

R. erythropolis cells contain a large set of important enzymes for bioconversion and biodegradation processes, which enable them to perform oxidations, dehydrogenations, epoxidations, hydrolysis, hydroxylations, dehalogenations, and desulfurizations (de Carvalho *et al.*, 2009). The exposure for 1 and 24 h respectively of *R. erythropolis* IBB_{Po1} cells to 1% alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) had different effects on their survival rate (Table 1). One hour after 1% alkanes and aromatics exposure the survival rates were 10^2-10^6 cfu mL^{-1}, and after 24 h the survival rates were 10^4-10^{10} cfu mL^{-1}. As anticipated, the viability of *R. erythropolis* IBB_{Po1} cells 1 and 24 h after 1% alkanes and aromatics exposure was lower, compared with the controls (10^7, 10^{11} cfu mL^{-1}). Tolerance of bacteria to organic solvents has been estimated by the solvent parameter log P_{OW}, which is an index of biological toxicity (Sikkema *et al.*, 1995). It is generally accepted that solvents with log P_{OW} values below 5 are considered extremely toxic because of their high degree of partitioning into the aqueous layer surrounding the cells, and from there into the lipid membrane bilayer (Torres *et al.*, 2011. However the cells that are able to remain viable after the first moments of contact with toxic organic solvents will be able to endure its presence for some time (de Carvalho *et al.*, 2009). In our study the results showed higher survival rates (10^5-10^{10} cfu mL^{-1}) when *R. erythropolis* IBB_{Po1} cells have been exposed to 1% al-

Table 1 - Viability of *R. erythropolis* IBB_{Po1} cells after 1% organic solvents exposure.

Cell viability[a]	Organic solvents (log P_{OW}[b])						
	Control	Cyclohexane (3.35)	*n*-Hexane (3.86)	*n*-Decane (5.98)	Toluene (2.64)	Styrene (2.86)	Ethylbenzene (3.17)
1 h	4.7×10^7	3.0×10^5	2.7×10^6	2.0×10^6	7.3×10^2	4.0×10^3	3.1×10^5
24 h	2.9×10^{11}	2.7×10^8	2.0×10^9	3.4×10^{10}	1.2×10^4	3.0×10^6	2.2×10^7

Legend: [a] = serial dilutions of cultures were spread on LB agar and the number of viable cells (cfu mL^{-1}) was determined; [b] = logarithm of the partition coefficient of the solvent in octanol-water mixture.

kanes (cyclohexane, *n*-hexane, *n*-decane) with log P_{OW} between 3.35 and 5.98, as compared with the survival rates of cells (10^2-10^7 cfu mL^{-1}) exposed to 1% aromatics (toluene, styrene, ethylbenzene) with log P_{OW} between 2.64 and 3.17. The survival rates drastically reduced from 10^7 and 10^{11} to 10^2 and 10^4, respectively, when *R. erythropolis* IBB_{Po1} cells were exposed to 1% toluene. This is in agreement with a previous study which found that organic solvents with lower log P_{OW} value (*e.g.*, benzene, toluene) bound more abundantly to bacterial cells thus being more toxic for them (Sikkema *et al.*, 1995; Torres *et al.*, 2011).

Cell morphology

Different adaptation responses of *R. erythropolis* IBB_{Po1} cells were observed by SEM (Figure 1a-1h) and TEM (Figure 1i-1p) studies, 1 and 24 h respectively after 1% alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) exposure. *R. erythropolis* IBB_{Po1} cells exposed to 1% alkanes and aromatics were free within the water phase as those in the control cells (Figure 1a), embedded in a polymeric layer (Figure 1b), closely grouped around polymeric structures of bacterial origin (Figure 1c, 1d), on the surface of biofilms (Figure 1e, 1f) or linked together as clusters (Figure 1g, 1h). All these structures were not observed in *R. erythropolis* IBB_{Po1} control cells. Similar results were pre-

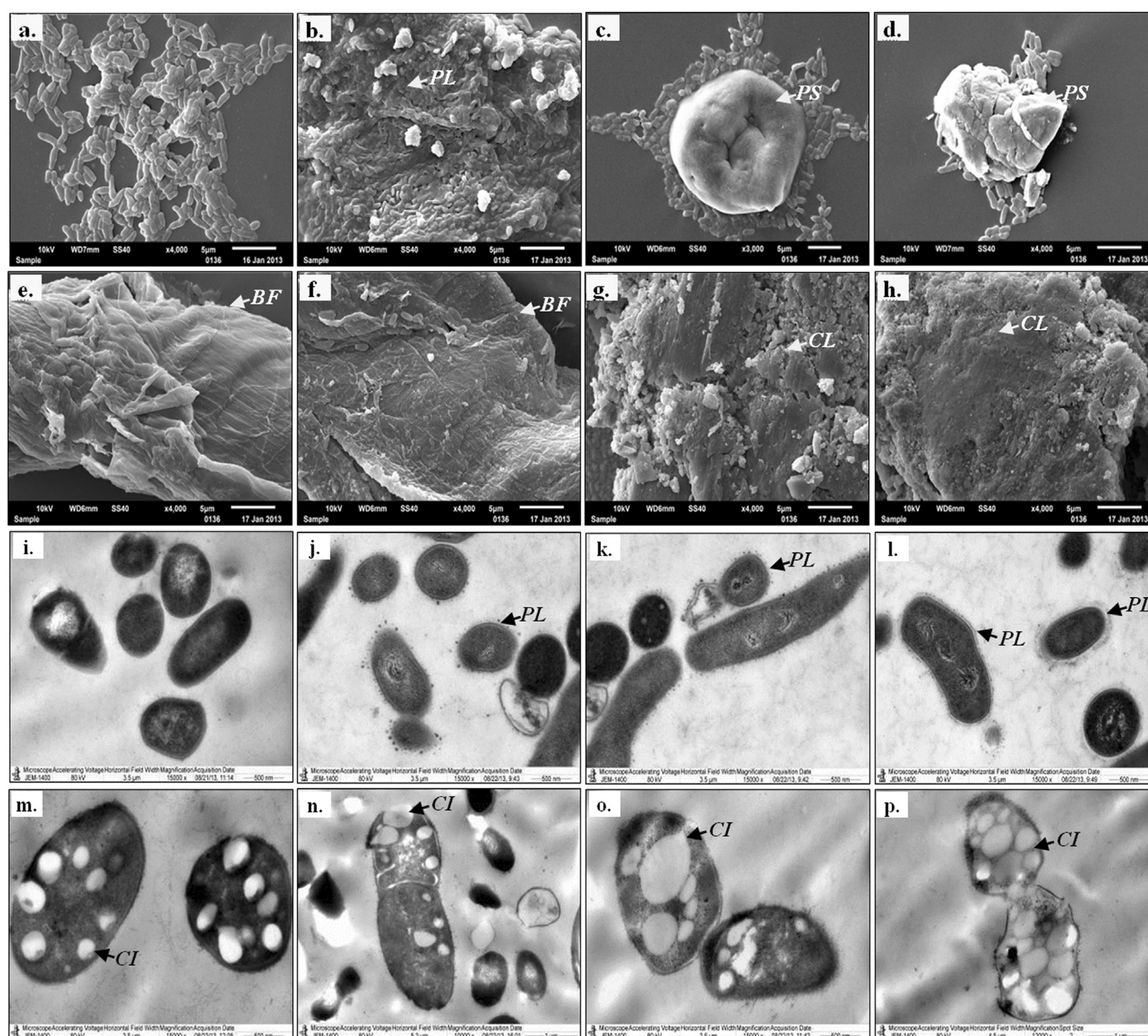

Figure 1 - SEM (panels a-h) and TEM (panels i-p) studies of *R. erythropolis* IBB_{Po1} cells after 1% organic solvents exposure. Bacterial cells cultivated 24 h in minimal medium (panels a-p); control (panels a, i); alkanes and aromatics (panels b-h, j-p); polymeric layers (PL), polymeric structures (PS), biofilms (BF), cells clusters (CL), cytoplasmatic electron-transparent inclusions (CI).

viously obtained by Rocha *et al.* (2011) for a Gram-negative bacterium *Pseudomonas aeruginosa* ATCC 55925 grown on 0.5% heating oil or pure alkanes (*i.e.*, C7-C18 *n*-alkanes, C19 branched alkane).

According to the literature (Urai *et al.*, 2007; Gutiérrez *et al.*, 2009; Rocha *et al.*, 2011; Torres *et al.*, 2011), the structures depicted only when the cells are exposed to hydrocarbons (*e.g.*, biofilms, cells clusters) play a significant role in toxic compounds tolerance and they protect the cells from different environmental stresses. Furthermore, the polymers produced by some bacterial strains play an important role in sequestering molecules of solvent (*i.e.*, benzene) within its immediate environment, thereby reducing solvent contact with its cell membrane and conferring it some degree of tolerance (Gutiérrez *et al.*, 2009).

In *R. erythropolis* IBB_{Po1} cells exposed to 1% alkanes (cyclohexane, *n*-hexane, *n*-decane), the cell membrane was intact (Figure 1j-1l) just like in the case of the control cells (Figure 1i), and no accumulation of solvents was seen in the cytoplasm. In cells exposed to 1% aromatics (toluene, styrene, ethylbenzene), the accumulation of solvent in the cytoplasm of cells with a disturbed cell membrane and an increase in the cell size were observed (Figure 1m-1p). The observed cytoplasmatic electron-transparent inclusions were similar to those reported previously for other bacterial strains, and their formation is a general cell adaptation response to hydrocarbon growth (Rocha *et al.*, 2011). When the large solvent inclusions occupied all the cytoplasm and the cell wall integrity was altered, the death of *R. erythropolis* IBB_{Po1} cells was also observed (Figure 1p).

Membrane permeability

In general, solvents exert their toxic effect by altering the cell membrane permeability (Gaur and Khare, 2009), which leads to the inactivation and denaturation of membrane-embedded proteins, and the promotion of leakages of ions and intracellular macromolecules, such as nucleic acids (Isken and de Bont, 1998). The exposure for 1 and 24 h of *R. erythropolis* IBB_{Po1} cells to 1% alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) had different effects on membrane permeability (Table 2).

The release of nucleic acids was higher after aromatics (0.509-0.834) and alkanes (0.277-0.370) exposure, compared with the controls (0.156, 0.234). Similar results were earlier obtained by Gaur and Khare (2009) for a Gram-negative bacterium *Pseudomonas aeruginosa* PseA grown in the presence of cyclohexane and tetradecane. These two organic solvents affect membrane integrity and structure dramatically, thereby altering the permeability and incurring toxicity. The higher release of nucleic acid into the growth medium when *R. erythropolis* IBB_{Po1} cells were grown in minimal medium in the presence of 1% aromatics (toluene, styrene, ethylbenzene) supports the results of TEM studies, which showed changes in the cytoplasmic membrane integrity of cells exposed to these toxic solvents.

Membrane lipids

Changes in the composition of phospholipids, glycolipids and mycolic acids of *R. erythropolis* have been suggested to depend on the availability and structure of the carbon source (de Carvalho *et al.*, 2009). The TLC analysis revealed the existence of some differences between phospholipids and glycolipids extracted from *R. erythropolis* IBB_{Po1} control cells and those extracted from cells exposed 1 and 24 h to 1% alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) (Figure 2a-2d).

The phospholipids (Figure 2a, 2b) found in *R. erythropolis* IBB_{Po1} control cells were identified based on their R_f (retardation factor) values as phosphatidylinositol (PI with R_f 0.45), cardiolipin (CL with R_f 0.75) and fatty acids (FA with R_f 0.79). The phosphatidylethanolamine (PE) was not detected in these extracts. Although the major lipid components of coryneform and nocardioform bacteria are cardiolipin and phosphatidylethanolamine (Kolomytseva *et al.*, 2005), only cardiolipin was detected in the *R. erythropolis* IBB_{Po1} cells extracts. The phospholipids found in *R. erythropolis* IBB_{Po1} cells 1 and 24 h after 1% alkanes and aromatics exposure were cardiolipin (with R_f 0.70-0.75) and fatty acids (with R_f 0.76-0.80). Phosphatidylinositol was detected only in barely detectable quantities in all extracts, except *n*-decane. An elevated level of cardiolipin was detected in extracts of *R. erythropolis*

Table 2 - Nucleic acid release by *R. erythropolis* IBB_{Po1} cells after 1% organic solvents exposure.

Cell permeability[a]	Organic solvents (log P_{OW}[b])						
	Control	Cyclohexane (3.35)	*n*-Hexane (3.86)	*n*-Decane (5.98)	Toluene (2.64)	Styrene (2.86)	Ethylbenzene (3.17)
1 h	0.156	0.277	0.244	0.257	0.509	0.567	0.597
24 h	0.234	0.362	0.370	0.333	0.834	0.778	0.695

[a]the absorbance of cell-free supernatant was measured at 260 nm; [b]logarithm of the partition coefficient of the solvent in octanol-water mixture.

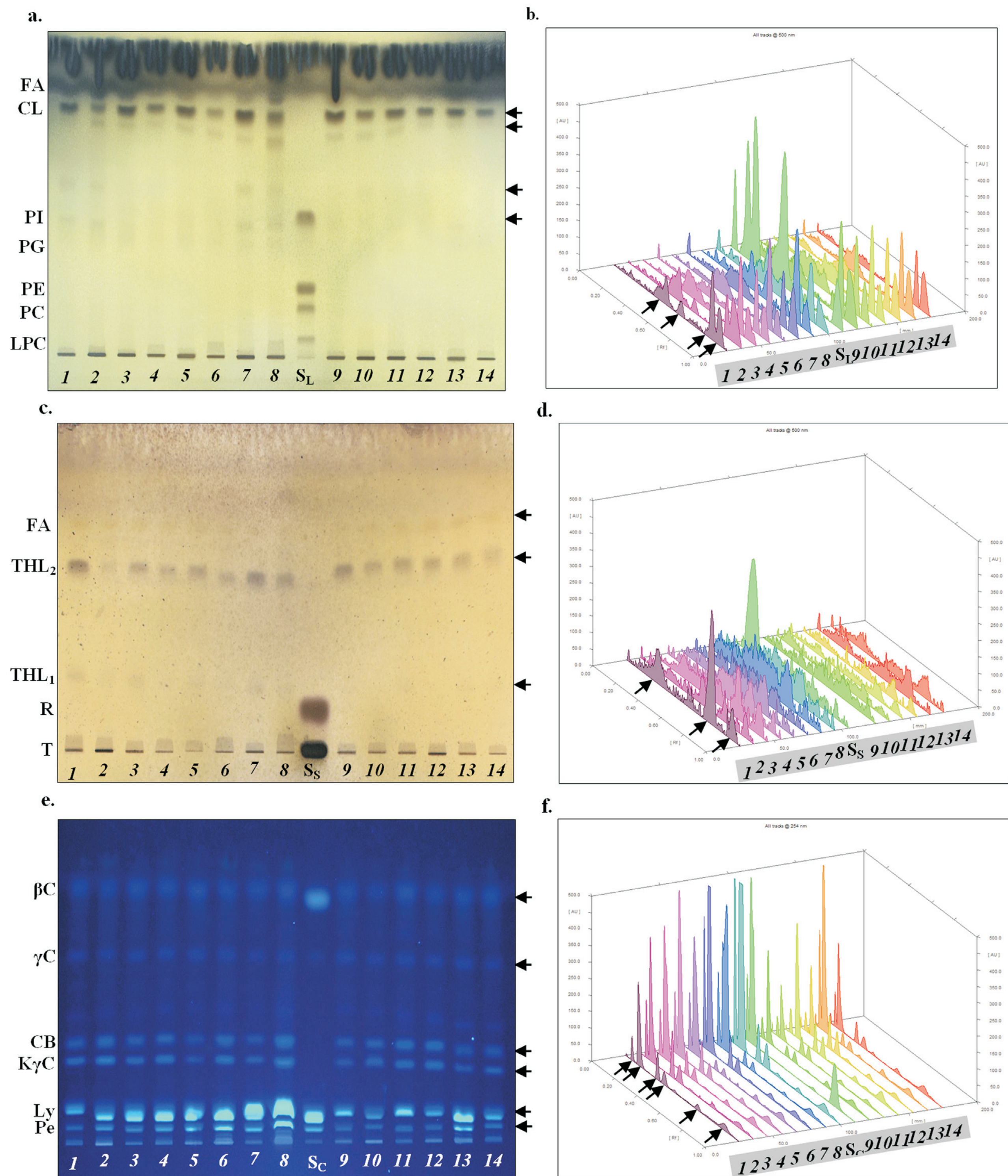

Figure 2 - Phospholipids (panels a, b), glycolipids (panels c, d) and carotenoids (panels e, f) of *R. erythropolis* IBB_{Po1} cells after 1% organic solvents exposure. The TLC plates were visualized (panels a, c, e) and scanned (panels b, d, f) under a 500 nm visible white light (panels a-d) or under a 254 nm ultraviolet light (panels e, f); bacterial cells cultivated 1 h (lanes 1, 3, 5, 7, 9, 11, 13) and 24 h (lanes 2, 4, 6, 8, 10, 12, 14) in minimal medium; control (lanes 1, 2), cyclohexane (lanes 3, 4), *n*-hexane (lanes 5, 6), *n*-decane (lanes 7, 8), toluene (lanes 9, 10), styrene (lanes 11, 12), ethylbenzene (lanes 13, 14). Panels a, b. Phospholipids standards (S_L), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), cardiolipin (CL), fatty acids (FA). Panels c, d. Sugars standards (S_S), trehalose (T), L-rhamnose (R), trehalolipids (THL_1, THL_2), fatty acids (FA). Panels e, f. Carotenoids standards (S_C), phytoene (Pe), lycopene (Ly or ψ, ψ-carotene), 4-keto-γ-carotene (KγC), chlorobactene (CB or Φ, ψ-carotene), γ-carotene (γC), β-carotene (βC). The phospholipids, glycolipids and carotenoids spots and their corresponding peak have been marked by arrows.

IBB_{Po1} cells 1 and 24 h after 1% alkanes and aromatics exposure (except styrene, ethylbenzene), as compared with those of the respective controls; no such changes were observed in cells exposed to styrene and ethylbenzene. It is known that phospholipids, as the major component of bacterial cells membranes, are responsible for their structural organization and selective permeability. Therefore, the increase in the content of cardiolipin and fatty acids in the membranes of cells cultured in the presence of the toxic compounds is the result of adaptation of the bacterial cells and has a defensive nature (Kolomytseva *et al.*, 2005; Martínková *et al.*, 2009).

The glycolipids (Figure 2c, 2d) found, based on their R_f values, in *R. erythropolis* IBB_{Po1} control cells were trehalolipids (THL_1 with R_f 0.30, THL_2 with R_f 0.61) and fatty acids (FA with R_f 0.72). The glycolipids found in *R. erythropolis* IBB_{Po1} cells 1 and 24 h after 1% alkanes and aromatics exposure were trehalolipids (THL_1 with R_f 0.26-0.29, THL_2 with R_f 0.57-0.63) and fatty acids (FA with R_f 0.70-0.74). THL_1 was not detected in extracts of cells exposed 1 h to toluene and for 24 h to cyclohexane, toluene and styrene. R_f values of THL_1 and THL_2 were similar to data given in the literature, and these value correspond to trehalose monomycolate and trehalose dimycolate, which are regular extractable components of the rhodococcal cell envelope (Niescher *et al.*, 2006).

An elevated level of THL_2 was detected in extracts of *R. erythropolis* IBB_{Po1} control cells, as compared with those of the cells exposed for 1 and 24 h to 1% alkanes and aromatics. This is not surprising because the trehalolipids could be released in high quantities into the growth medium when the cells are exposed to 1% organic solvents, as compared with the control cells. We observed previously that *R. erythropolis* IBB_{Po1} is a good biosurfactant producer (emulsification index E_{24} = 100%), compared with other *Rhodococcus* strains (Stancu, 2014). The biosurfactants released into the growth medium modify the cell surface hydrophobicity and/or promote emulsification and/or solubilization of organic solvents thus accelerating their biodegradation (Philp *et al.*, 2002; Gesheva *et al.*, 2010). Such solvent-tolerant bacteria provide the key for the use of otherwise toxic solvents in whole-cell two-phase biotransformations, by overcoming the toxic effects of substrates and products (Heipieper *et al.*, 2007).

Carotenoid pigments

Carotenoids are hydrophobic molecules typically associated with cytoplasmic membrane and/or noncovalently bound to specific proteins. These pigments form an integral part of the complex membrane structure of some bacteria and influence membrane fluidity, by increasing its rigidity and mechanical strength (Godinho and Bhosle, 2008). It has been suggested that the presence of carotenoids may change the effectiveness of the membrane as a barrier to water, oxygen, and other molecules. Some bacteria may be accumulating carotenoids as part of their responses to various environmental stresses, and thus aiding their survival in this habitat (Godinho and Bhosle, 2008). Therefore, we further investigated the effect of the 1% alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) to the carotenoids synthesis in *R. erythropolis* IBB_{Po1} cells. The UV/visible absorption scanning spectra of the pigment extract of *R. erythropolis* IBB_{Po1} cells showed absorption maxima at 340 nm. Carotenoid pigments extracted from *R. erythropolis* IBB_{Po1} cells 1 and 24 h after 1% alkanes and aromatics exposure showed the same absorption maxima. The TLC plate developed with chloroform-methanol mixture showed the separation of the carotenoid pigments into 6 fluorescent spots (Figure 2e). The carotenoids synthesized by different *R. erythropolis* strains were previously characterized to be 4-keto-γ-carotene as the major carotenoid and sometimes γ-carotene as the minor carotenoids (Tao *et al.*, 2006). However, other carotenoids (*e.g.*, phytoene, lycopene, chlorobactene, β-carotene) were also described in *R. erythropolis* (Tao *et al.*, 2006). The carotenoids (Figure 2e, 2f) found, based on their R_f values, in *R. erythropolis* IBB_{Po1} control cells were phytoene (Pe with R_f 0.04), lycopene (Ly or ψ, ψ-carotene with R_f 0.10), 4-keto-γ-carotene (KγC with R_f 0.23), chlorobactene (CB or Φ, ψ-carotene with R_f 0.29), γ-carotene (γC with R_f 0.53), β-carotene (βC with R_f 0.73). The carotenoids found in *R. erythropolis* IBB_{Po1} cells 1 and 24 h after 1% alkanes and aromatics exposure were phytoene (with R_f 0.04-0.06), lycopene (with R_f 0.07-0.10), 4-keto-γ-carotene (with R_f 0.20-0.24), chlorobactene (with R_f 0.26-0.30), γ-carotene (with R_f 0.51-0.53), β-carotene (with R_f 0.71-0.73). An elevated level of lycopene (which is an important intermediate in the biosynthesis of γ-carotene and β-carotene) was detected in extracts of *R. erythropolis* IBB_{Po1} cells 1 and 24 h after 1% alkanes exposure (especially in the case of *n*-decane), as compared with those of the control cells; no such changes were observed in cells exposed to 1% aromatics (except ethylbenzene).

16S rRNA gene expression

Because rhodococci commonly exhibit considerable genomic instabilities that can be specifically selected (Larkin *et al.*, 2006), we further investigated the expression of 16S rRNA gene after *R. erythropolis* IBB_{Po1} cells exposure to organic solvents. Genomic DNA extracted from R. *erythropolis* IBB_{Po1} control cells and those extracted from cells 1 and 24 h after 1% alkanes and aromatics exposure was used as template for PCR amplification of 16S rRNA gene. The expected PCR product size was 1465 bp for this gene (Figure 3a).

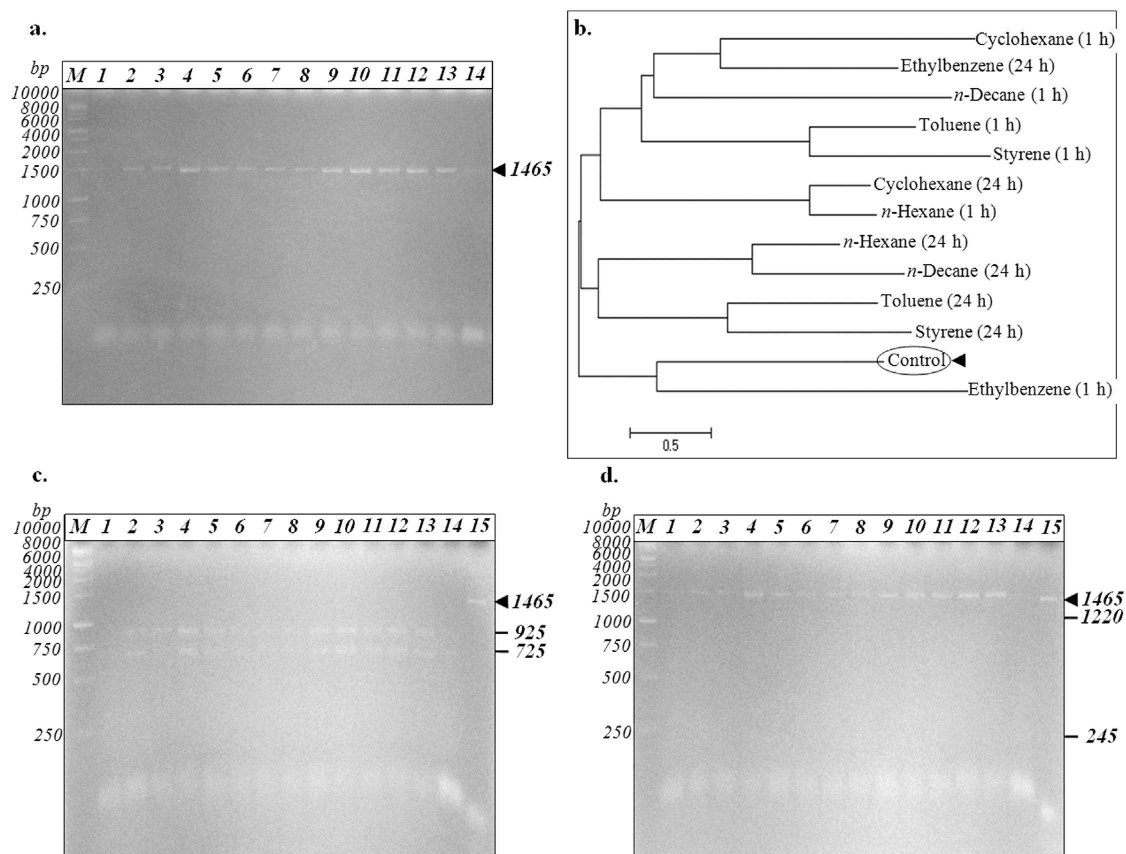

Figure 3 - Detection of 16S rRNA gene (panels a, c, d) and phylogenetic tree based on 16S rRNA gene sequences (panel b) of *R. erythropolis* IBB_{Po1} cells after 1% organic solvents exposure. Panel a. 16S rRNA gene; Panel c. 16S rRNA gene digested with restriction endonuclease *Eco*RI; Panel d. 16S rRNA gene digested with restriction endonuclease *XbaI*; bacterial cells cultivated 1 h (lanes 1, 3, 5, 7, 9, 11, 13) and 24 h (lanes 2, 4, 6, 8, 10, 12, 14) in minimal medium; control (lanes 1, 2), cyclohexane (lanes 3, 4), *n*-hexane (lanes 5, 6), *n*-decane (lanes 7, 8), toluene (lanes 9, 10), styrene (lanes 11, 12), ethylbenzene (lanes 13, 14); undigested 16S rRNA gene (lane 15); 1 kb DNA ladder (lane *M*). Panel b. The phylogenetic tree was obtained using the neighbor-joining method in MEGA5.1 program. The scale bar indicates substitutions per nucleotide position.

PCR product of 16S rRNA gene (1465 bp fragment) from *R. erythropolis* IBB_{Po1} cells was digested by *Eco*RI and *Xba*I restriction endonucleases (Figure 3c, 3d). The *Eco*RI recognition site exists in the sequence of 16S rRNA gene from *R. erythropolis* IBB_{Po1}, while *Xba*I recognition site did not exist in this bacterium. When 16S rRNA gene from *R. erythropolis* IBB_{Po1} cells (control) was digested by *Eco*RI, two distinct bands (925+725 bp) were observed (Figure 3c). The same RFLP pattern was obtained for *R. erythropolis* IBB_{Po1} cells exposed 1 and 24 h to 1% alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene).

In the 16S rRNA phylogenetic tree obtained using the neighbor-joining method (Figure 3b), control (KF059972.1) formed a tight cluster with only 16S rRNA gene sequence of the cells exposed 1 h to ethylbenzene. Moreover, considerable modifications in 16S rRNA gene sequence of *R. erythropolis* IBB_{Po1} cells were observed 24 h after ethylbenzene exposure, 1 and 24 h after 1% alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene) exposure, as compared with the control (Figure 3b). It is not surprising to observe DNA sequence modification after organic solvent exposure, because these toxic compounds are metabolically activated in cells to yield highly reactive bay region dihydrodiol epoxide derivatives which can effectively attack DNA, leading to disruption of normal cellular functions (Wei *et al.*, 1984).

Conclusions

Solvent-tolerant bacteria belonging to the genus *Rhodococcus* are increasingly recognized as very good candidates for the biodegradation of toxic compounds, because of their ability to degrade a wide range of organic compounds, hydrophobic cell surfaces, biosurfactant production, and ubiquity and robustness in the environment (Pini *et al.*, 2007; Martinkova *et al.*, 2009; Torres *et al.*, 2011).

Organic solvents with log P_{OW} values below 5 are considered extremely toxic to bacteria. However we have previously shown that *R. erythropolis* IBB_{Po1} cells had a

good tolerance (40-100%) to both alkanes (cyclohexane, n-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) with log P_{OW} values between 2.64 and 5.98. Additionally, *R. erythropolis* IBB_{Po1} was found to utilize these toxic organic solvents as the sole carbon source. Modifications in the cell viability, cell morphology, membrane permeability, lipid profile, carotenoid pigments profile and 16S rRNA gene were revealed in *R. erythropolis* IBB_{Po1} cells grown 1 and 24 h on minimal medium in the presence of 1% alkanes (cyclohexane, n-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene). The acquired results showed higher survival rates when *R. erythropolis* IBB_{Po1} cells were exposed to 1% alkanes (cyclohexane, *n*-hexane, *n*-decane) with log P_{OW} between 3.35 and 5.98, compared with those of the cells exposed to 1% aromatics (toluene, styrene, ethylbenzene) with log P_{OW} between 2.64 and 3.17. Due to its environmental origin and its metabolic potential, *R. erythropolis* IBB_{Po1} is an excellent candidate for bioremediation of soils contaminated with crude oils and other toxic compounds. Bioremediation of soil contaminated with 5% (w/v) Poeni crude oil was studied for a period of 30 days, under laboratory condition (data not shown). The amount of crude oil degraded by *R. erythropolis* IBB_{Po1} after 15 and 30 days incubation were 34% and 85%, respectively. Moreover, the carotenoids produced by this nonpathogenic Gram-positive bacterium can have a variety of other potential applications (medicine, cosmetics, food industry).

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