Response of *Rhodococcus erythropolis* strain IBBp01 to toxic organic solvents

Mihaela Marilena Stancu
Institute of Biology Bucharest of Romanian Academy, Bucharest, Romania.

Submitted: May 29, 2014; Approved: March 11, 2015.

**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* IBBp01 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* IBBp01 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* IBBp01 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* IBBp01 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; Martinková 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).
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\textsubscript{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 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\textsubscript{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\textsubscript{Po1} (KF059972.1) within the genus *Rhodococcus*, showing 95% similarity to other *Rhodococcus* strains from the public databases (GenBank/DBJ/EMBL).

**Response of *R. erythropolis* IBB\textsubscript{Po1} cells to organic solvents**

Bacterial cells were cultivated on liquid LB medium containing 30 $\mu$g 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) $\alpha$-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 SPECTORD 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 $\mu$L of DNA extract was added to a final volume of 50 $\mu$L 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.
Response of R. erythropolis to solvents

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 IBBPo1 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, hydrolyses, hydroxylations, dehalogenations, and desulphurizations (de Carvalho et al., 2009).

Results and Discussion

Response of R. erythropolis IBBPo1 cells to organic solvents

Numerous and complex physiological cellular responses and adaptations involved in organic solvents tolerance have been observed in R. erythropolis IBBPo1 cells grown on LB medium in the presence of 1% organic solvents (Stancu, 2014). This bacterium was adapted 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 IBBPo1 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.

Table 1 - Viability of R. erythropolis IBBPo1 cells after 1% organic solvents exposure.

| Cell viability | Organic solvents (log $P_{OW}$) |
|---------------|---------------------------------|
|               | 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 x 10^7                    | 3.0 x 10^7         | 2.7 x 10^6      | 2.0 x 10^6      | 7.3 x 10^2      | 4.0 x 10^7      | 3.1 x 10^7          |
| 24 h          | 2.9 x 10^11                   | 2.7 x 10^9         | 2.0 x 10^9      | 3.4 x 10^9      | 1.2 x 10^4      | 3.0 x 10^6      | 2.2 x 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 IBBPo1 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 IBBPo1 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 IBBPo1 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 IBBPo1 control cells. Similar results were pre-

![Figure 1](image-url)  
**Figure 1** - SEM (panels a-h) and TEM (panels i-p) studies of \(R.\) erythropolis IBBPo1 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* IBBPo1 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* IBBPo1 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* IBBPo1 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* IBBPo1 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* IBBPo1 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* IBBPo1 control cells were identified based on their *R*<sub>f</sub> (retardation factor) values as phosphatidylinositol (PI with *R*<sub>f</sub> 0.45), cardiolipin (CL with *R*<sub>f</sub> 0.75) and fatty acids (FA with *R*<sub>f</sub> 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* IBBPo1 cells extracts. The phospholipids found in *R. erythropolis* IBBPo1 cells 1 and 24 h after 1% alkanes and aromatics exposure were cardiolipin (with *R*<sub>f</sub> 0.70-0.75) and fatty acids (with *R*<sub>f</sub> 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*

| Cell permeability<sup>a</sup> | 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             |

<sup>a</sup>the absorbance of cell-free supernatant was measured at 260 nm; <sup>b</sup>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* IBBPn1 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<sub>L</sub>), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), cardiolipin (CL), fatty acids (FA). Panels c, d. Sugars standards (S<sub>S</sub>), trehalose (T), L-rhamnose (R), trehalolipids (THL<sub>L1</sub>, THL<sub>L2</sub>), fatty acids (FA). Panels e, f. Carotenoids standards (S<sub>C</sub>), phytoene (Pe), lycopene (Ly or ψ-carotene), 4-keto-β-carotene (KyC), 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.
IBBPo1 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 cell 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; Martinková et al., 2009).

The glycolipids (Figure 2c, 2d) found, based on their Rf values, in R. erythropolis IBBPo1 control cells were trehalolipids (THL1 with Rf 0.30, THL-2 with Rf 0.61) and fatty acids (FA with Rf 0.72). The glycolipids found in R. erythropolis IBBPo1 cells 1 and 24 h after 1% alkanes and aromatics exposure were trehalolipids (THL1 with Rf 0.26-0.29, THL2 with Rf 0.57-0.63) and fatty acids (FA with Rf 0.70-0.74). THL1 was not detected in extracts of cells exposed 1 h to toluene and for 24 h to cyclohexane, toluene and styrene. Rf values of THL1 and THL2 were similar to data given in the literature, and these values 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 IBBPo1 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 IBBPo1 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; Geshova 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 IBBPo1 cells. The UV/visible absorption scanning spectra of the pigment extract of R. erythropolis IBBPo1 cells showed absorption maxima at 340 nm. Carotenoid pigments extracted from R. erythropolis IBBPo1 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-\(\gamma\)-carotene as the major carotenoid and sometimes \(\gamma\)-carotene as the minor carotenoids (Tao et al., 2006). However, other carotenoids (e.g., phytoene, lycopene, chlorobactene, \(\beta\)-carotene) were also described in R. erythropolis (Tao et al., 2006). The carotenoids (Figure 2e, 2f) found, based on their Rf values, in R. erythropolis IBBPo1 control cells were phytoene (Pe with Rf 0.04), lycopene (Ly or \(\psi\), \(\psi\)-carotene with Rf 0.10), 4-keto-\(\gamma\)-carotene (K\(\gamma\)C with Rf 0.23), chlorobactene (CB or \(\Phi\), \(\psi\)-carotene with Rf 0.29), \(\gamma\)-carotene (\(\gamma\)C with Rf 0.53), \(\beta\)-carotene (BC with Rf 0.73). The carotenoids found in R. erythropolis IBBPo1 cells 1 and 24 h after 1% alkanes and aromatics exposure were phytoene (with Rf 0.04-0.06), lycopene (with Rf 0.07-0.10), 4-keto-\(\gamma\)-carotene (with Rf 0.20-0.24), chlorobactene (with Rf 0.26-0.30), \(\gamma\)-carotene (with Rf 0.51-0.53), \(\beta\)-carotene (with Rf 0.71-0.73). An elevated level of lycopene (which is an important intermediate in the biosynthesis of \(\gamma\)-carotene and \(\beta\)-carotene) was detected in extracts of R. erythropolis IBBPo1 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 IBBPo1 cells exposure to organic solvents. Genomic DNA extracted from R. erythropolis IBBPo1 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).
PCR product of 16S rRNA gene (1465 bp fragment) from *R. erythropolis IBBPo1* 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* IBBPo1, while *Xba*I recognition site did not exist in this bacterium. When 16S rRNA gene from *R. erythropolis* IBBPo1 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* IBBPo1 cells exposed 1 and 24 h to 1% alkanes (*cyclohexane, n-hexane, n-decane*) and aromatics (*toluene, styrene*). In the 16S rRNA phylogenetic tree obtained using the neighbor-joining method in MEGA5.1 program, control (lanes 1, 2) and 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.

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 IBBPo1* 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 IBBP01 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 IBBP01 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 IBBP01 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 IBBP01 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 IBBP01 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).

**Acknowledgments**

This study was funded by project no. RO1567-IBB05/2014 from the Institute of Biology Bucharest of Romanian Academy. The author is grateful to Ana Dinu and Alexandru Brînzan for technical support.

**References**

Altschul SF, Gish W, Miller W et al. (1990) Basic local alignment search tool. J Mol Biol 215:403-410.

Das P, Mukherjee S, Sen R (2009) Substrate dependent production of extracellular biosurfactant by a marine bacterium. Biore Technol 100:1015-1019.

de Carvalho CCCR, Wick L-Y, Heipieper HJ (2009) Cell wall adaptations of planktonic and biofilm *Rhodococcus erythropolis* cells to growth on C5 to C16 n-alkane hydrocarbons. Appl Microbiol Biotechnol 82:311-320.

Gaur R, Khare SK (2009) Cellular response mechanisms in *Pseudomonas aeruginosa* PseA during growth in organic solvents. Lett Appl Microbiol 49:372-377.

Gesheva V, Stackebrandt E, Vasileva-Tonkova E (2010) Biosurfactant production by halotolerant *Rhodococcus fascians* from Casey Station, Wilkes Land, Antarctica. Curr Microbiol 61:112-117.

Godinho A, Bhosale S (2008) Carotenes produced by alkapilpic orange-pigmented strain of *Microbacterium arborescens* - AGSB isolated from coastal sand dunes. Indian J Mar Sci 37:307-312.

Gutiérrez T, Learmonth R, Couperwhite I (2009) Analysis of benzene-induced effects on Rhodococcus sp. 33 reveals that constitutive processes play a major role in conferring tolerance. Sci World J 9:209-223.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95-98.

Heipieper HJ, Neumann G, Cornelissen S et al. (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl Microbiol Biotechnol 74:961-973.

Isken S, de Bont JAM (1998) Bacteria tolerant to organic solvents. Extremophiles 2:229-238.

Kolomytseva MP, Solyanikova IP, Golovlev EL et al. (2005) Heterogeneity of Rhodococcus opacus 1CP as a response to stress induced by chlorophenols. Appl Biochem Microbiol 41:474-479.

Larkin MJ, Kulakov LA, Allen CCR (2006) Biodegradation by members of the genus *Rhodococcus*: biochemistry, physiology, and genetic adaptation. Adv Appl Microbiol 59:1-29.

Marchesi JR, Sato T, Weightman AJ et al. (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64:795-799.

Martínková L, Uhnháková B, Pátek M et al. (2009) Biodegradation potential of the genus *Rhodococcus*. Environ Int 35:162-177.

Niescher S, Wray V, Lang S et al. (2006) Identification and structural characterisation of novel trehalose dinocardio-mycolates from n-alkane-grown *Rhodococcus opacus* 1CP. Appl Microbiol Biotechnol 70:605-611.

Phlip JC, Kuyukina MS, Ishihina IB et al. (2002) Alkanotrophic *Rhodococcus ruber* as a biosurfactant producer. Appl Microbiol Biotechnol 59:318-324.

Pini F, Grossi C, Nereo S et al. (2007) Molecular and physiological characterisation of psychrophilic hydrocarbon-degrading bacteria isolated from Terra Nova Bay (Antarctica). Eur J Soil Biol 43:368-379.

Rocha CA, Pedregosa AM, Laborda F (2011) Biosurfactant-mediated biodegradation of straight and methyl-branched alkanes by *Pseudomonas aeruginosa* ATCC 55925. AMB Express 1:1-10.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning, A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Segura A, Hurtado A, Rivera B et al. (2008) Isolation of new toluene-tolerant marine strains of bacteria and characterization of their solvent-tolerance properties. J Appl Microbiol 104:1408-1416.

Sikkema J, de Bont JAM, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201-222.

Stancu MM, Grifoll M (2011) Multidrug resistance in hydrocarbon-tolerant Gram-positive and Gram-negative bacteria. J Gen Appl Microbiol 57:1-18.

Stancu MM (2014) Physiological cellular responses and adaptations of *Rhodococcus erythropolis* IBBP01 to toxic organic solvents. J Environ Sci 26:in press.
Tamura K, Peterson D, Peterson N et al. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molec Biol Evol 28:2731-2739.

Tao L, Wagner LW, Rouvière PE et al. (2006) Metabolic engineering for synthesis of aryl carotenoids in Rhodococcus. Appl Microbiol Biotechnol 70:222-228.

Torres S, Pandey A, Castro GR (2011) Organic solvent adaptation of Gram positive bacteria: applications and biotechnological potentials. Biotechnol Adv 29:442-452.

Urai M, Yoshizaki H, Anzai H et al. (2007) Structural analysis of an acidic, fatty acid ester-bonded extracellular polysaccharide produced by a pristine assimilating marine bacterium, Rhodococcus erythropolis PR4. Carbohydr Res 342:933-942.

Wei SJC, Desai SM, Harvey RG et al. (1984) Use of short DNA oligonucleotides for determination of DNA sequence modifications induced by benzo[a]pyrene diol epoxide. Proc Natl Acad Sci USA 81:5936-5940.

Whyte LG, Greer CW, Inniss WE (1996) Assessment of the biodegradation potential of psychrotrophic microorganisms. Can J Microbiol 42:99-106.

Yoo M, Kim D, Choi KY et al. (2012) Draft genome sequence and comparative analysis of the superb aromatic-hydrocarbon degrader Rhodococcus sp. strain DK17. J Bacteriol 194:4440.

Zhang Y, Qin F, Qiao J et al. (2012) Draft genome sequence of Rhodococcus sp. strain P14, a biodegrader of high-molecular-weight polycyclic aromatic hydrocarbons. J Bacteriol 194:3546.

Associate Editor: Valeria Maia de Oliveira

All the content of the journal, except where otherwise noted, is licensed under a Creative Commons License CC BY-NC.