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ORGANIC SOLVENT RESISTANCE MECHANISMS IN *RHODOCOCCUS* sp. Po_4

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Abstract

Crude oil extraction, transportation and storage facilities are frequently the source of soil and water pollution in Romania. Rhodococcus sp. Po_4 showed good tolerance to both 1 % (v/v) alkanes (cyclohexane, n-hexane, n-decane) and aromatics (toluene, styrene, ethylbenzene) with log P_{OW} (logarithm of the partition coefficient of the solvent in n-octanol-water mixture) values between 2.64 and 5.98. However, 1 % (v/v) alkanes were less toxic for Rhodococcus sp. Po_4 cells, compared with 1 % (v/v) aromatics. The high organic solvent tolerance of Rhodococcus sp. Po_4 could be due to the presence in their large genome of some catabolic and trehalose-6-phosphate synthase genes. In addition, Rhodococcus sp. Po_4 exhibits potential to synthesize carotenoid pigments which can improve the cell membrane impermeability to toxic organic solvents.

Keywords: *mechanisms, resistance, Rhodococcus, organic solvents.*

INTRODUCTION

The genus *Rhodococcus* belongs to mycolic-acid containing actinomycetes. Many bacteria from the order *Actinomycetes* are known to be antibiotic producers (e.g., *Streptomyces*), while rhodococci are known to exhibit a broad range of catalytic activity that has applications in industrial, pharmaceutical, and environmental biotechnology (Kagawa et al., 2013). Strains of *Rhodococcus* have been reported to be particularly solvent tolerant, while presenting a broad array of enzymes with potential for the production of commercially interesting compounds and/or for the metabolism of recalcitrant organic solvents. The adaptability and versatility of *Rhodococcus* cells can further broaden their application scope (de Carvalho, 2010).

Several reviews have been published on the tolerance of bacterial cells to organic solvents in which the mechanisms of cell adaptation were discussed (Sikkema et al., 1995; de Carvalho, 2010; Torres et al., 2011; Segura et al., 2012). Most of the papers published on this subject dealt with Gram-negative bacteria. Although the interest in using Gram-positive bacteria in biocatalysis and bioremediation processes is increasing, studies on the adaptation of *Rhodococcus* strains to toxic organic solvents are still scarce (de Carvalho, 2010; Torres et al., 2011). *Rhodococcus* cells can adapt the cell wall and membrane compositions, as well as the physicochemical properties of the cell surface, can degrade or bioconvert toxic compounds (e.g., benzene, toluene), and can aggregate and produce exopolymeric substances to protect the cell population from stressful environments (de Carvalho, 2010).

The possible cell adaptation mechanisms pursued by *Rhodococcus* sp. Po_4 after 1 % (v/v) alkanes and aromatics exposure was studied by following the changes in the cell viability, carotenoid pigments production and in their genomic fingerprinting.

MATERIALS AND METHODS

Organic solvent resistance mechanisms in *Rhodococcus* sp. Po_4. This bacterial strain was cultivated on liquid LB-Mg medium (Stancu and Grifoll, 2011) and incubated at 28 °C on a rotary shaker (200 rpm) until they reached a turbidity of 0.600 (OD_{660nm}). Then, 1 % (v/v) organic solvents (alkanes: cyclohexane, *n*-hexane, *n*-decane; aromatics: toluene, styrene, ethylbenzene) were supplied to the culture broths. Flasks were sealed and incubated for 1 and 24 hours at 28 °C on a rotary shaker (200 rpm).

Cell viability. Serial dilutions of the culture broths were spotted on LB-Mg agar and the number of viable cells (CFU ml^{-1}) was determined after 24 hours incubation of the Petri plates at 28 °C.

Pigments production. Culture broths were spotted on LB-Mg agar and the pigments production was observed after 24 hours incubation of the Petri plates at 28 °C. The Petri plates were visualized under a 254 nm ultraviolet light, and the fluorescence of the cultures is due to carotenoid pigments. Carotenoids were extracted from the culture broths with acetone. UV/visible scanning spectra of the samples were recorded between 220 and 700 nm using a NanoDrop1000 UV-visible spectrophotometer. For HPTLC analysis, the samples were spotted with a Linomat 5 sample applicator (CAMAG), on a 10×20 cm precoated silica gel 60 TLC aluminium sheets (Merck). The separation was performed using acetone-*n*-hexane (7.5:92.5 v/v) mixture (Tao et al., 2004) as mobile phase. The TLC plates were visualized under a 254 nm ultraviolet light.

Genomic fingerprinting. DNA from culture broths was isolated using the method of Whyte et al. (1996). For rep-PCR (repetitive sequence-based PCR) amplification, 1 µl of DNA extract was added to a final volume of 25 µl reaction mixture, containing: 5×GoTaq flexi buffer, $MgCl_2$, dNTP mix, specific primers (REP 1R-Dt and REP 2-Dt, BOXA 1R, Versalovic, et al. 1994), and GoTaq DNA polymerase (Promega). PCR was performed with a mastercycler proS (Eppendorf). The PCR program consisted in initial denaturation for 6 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 40 to 50 °C for 1 min, extension at 65 °C for 8 min, and a final extension at 65 °C for 16 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 rep-PCR products were analyzed.

Reagents used during this study were procured from Merck, Sigma-Aldrich, Promega, Invitrogen, Zymo Research, Applied Biosystems, Biolog or Bio-Rad Laboratories. The PCR primers were purchased from Biosearch Technologies, Integrated DNA Technologies and Invitrogen.

RESULTS AND DISCUSSIONS

Rhodococci are often isolated from soils contaminated with crude oil and/or xenobiotic compounds, and these organisms can utilize such compounds as carbon sources (Kagawa et al., 2013). The strain used in the present study was *Rhodococcus* sp. Po_4. This nonpathogenic Gram-positive bacterium was isolated previously from Poeni oily sludge by enrichment cultures method (Stancu and Grifoll, 2011). Their taxonomic affiliation was determined on the basis of phenotypic characteristics, G+C content of the bacterial chromosome, and the 16S rRNA gene sequence (Stancu and Grifoll, 2011). In the 16S rRNA gene phylogenetic tree obtained using the neighbour-joining method in MEGA5.1 program (Tamura et al., 2011), *Rhodococcus* sp. Po_4 formed a cluster with four strains of *Rhodococcus qingshengii* (Ba49, PT3-14, PT2-14B, BLH-Y4) and two strains of *Rhodococcus* sp. (Y2-2-10, G1-2-10) (Figure 1).

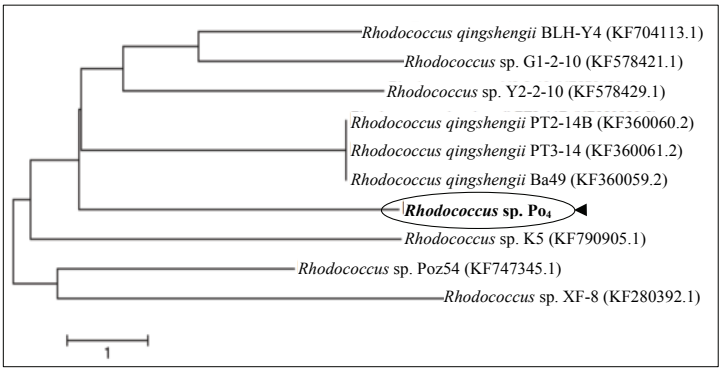

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of *Rhodococcus* sp. Po_4 with respect to other *Rhodococcus* strains from GenBank public database
The scale bar indicates substitutions per nucleotide position.

Organic solvent resistance mechanisms in *Rhodococcus* sp. Po$_4$.

Cell viability. The exposure for 1 and 24 hours of *Rhodococcus* sp. Po$_4$ cells to 1 % (v/v) alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) had different effects on their survival rate (Table 1).

Table 1. Viability of *Rhodococcus* sp. Po$_4$ cells after 1 % (v/v) 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 hour	2.7×10^{9}	3.3×10^{7}	4.7×10^{8}	2.0×10^{8}	4.0×10^{5}	8.3×10^{6}	1.2×10^{7}
24 hours	3.1×10^{12}	2.2×10^{10}	1.7×10^{11}	2.3×10^{11}	4.3×10^{7}	1.4×10^{8}	2.1×10^{9}

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

The viability of *Rhodococcus* sp. Po$_4$ cells 1 and 24 hours after alkanes and aromatics exposure was lower (10^5-10^{11} CFU ml^{-1}), compared with the controls (10^9, 10^{12} CFU ml^{-1}). One hour after alkanes and aromatics exposure the survival rates were 10^5-10^8 CFU ml^{-1}, and after 24 hours the survival rates were 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). In our study the results showed higher survival rates (10^7-10^{11} CFU ml^{-1}) when *Rhodococcus* sp. Po$_4$ cells were exposed to alkanes (cyclohexane, *n*-hexane, *n*-decane) with log P_{OW} between 3.35 and 5.98, as compared with the survival rates of cells (10^5-10^9 CFU ml^{-1}) exposed to aromatics (toluene, styrene, ethylbenzene) with log P_{OW} between 2.64 and 3.17. This is in agreement with a previous study which found that organic solvents with lower log P_{OW} value bound more abundantly to bacterial cells thus being more toxic for them (Sikkema et al., 1995; Torres et al., 2011).

The high organic solvent tolerance of *Rhodococcus* sp. Po$_4$ could be due to the presence in their large genome of some catabolic (i.e., *alkB/alkB1*) (Stancu and Grifoll, 2011) and trehalose-6-phosphate synthase (*otsA1*) genes. The DNA extracted from *Rhodococcus* sp. Po$_4$ was screened by PCR for the presence of *otsA1* gene using otsA-f and otsA-r degenerate primers (Tischler et al., 2013). PCR amplification was performed as described by Tischler et al. (2013). As expected, *Rhodococcus* sp. Po$_4$ possesses the *otsA1* gene (760 bp). According to literature (Tischler et al., 2013), this gene seems to be involved in the overproduction of trehalose lipids by *Rhodococcus opacus* 1CP during growth on different *n*-alkanes.

Pigments production. It is well known that the carotenoid pigments are present in some bacteria, and they have important functions in photosynthesis, nutrition, and protection against oxidative damage (Tao et al., 2004). Some natural carotenoids are asymmetrical molecules that are difficult to produce chemically, and the biological production of carotenoids using specific enzymes is a potential alternative to extraction from natural sources (Tao et al., 2004). As observed in Figures 2a-2c, *Rhodococcus* sp. Po$_4$ exhibits potential to synthesize carotenoid pigments. Therefore, we further investigated the effect of 1 % (v/v) alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) to the carotenoids in *Rhodococcus* sp. Po$_4$ cells.

On the Petri plate, no significant modifications in pigments production was observed in *Rhodococcus* sp. Po$_4$ cells 1 and 24 hours after alkanes and aromatics (except toluene) exposure, as compared with the controls (Figure 2a). The fluorescence of the cultures was similar in control cells and those of the cells exposed 1 and 24 hours to alkanes and aromatics. Only in the case of the cells exposed to toluene the fluorescence of the cultures was lower compared with the controls.

The carotenoids synthesized by different *Rhodococcus* strains were previously (Tao et al., 2004) characterized to be 4-keto-γ-carotene (KγC), γ-carotene (γC), chlorobactene (CB) and β-carotene (βC). The carotenoids (Figure 2b) found, based on their R_f values, in *Rhodococcus* sp. Po_4 cells were 4-keto-γ-carotene (with R_f 0.03-0.06), chlorobactene (with R_f 0.09-0.11), γ-carotene (with R_f 0.19-0.21), and β-carotene (with R_f 0.37-0.42).

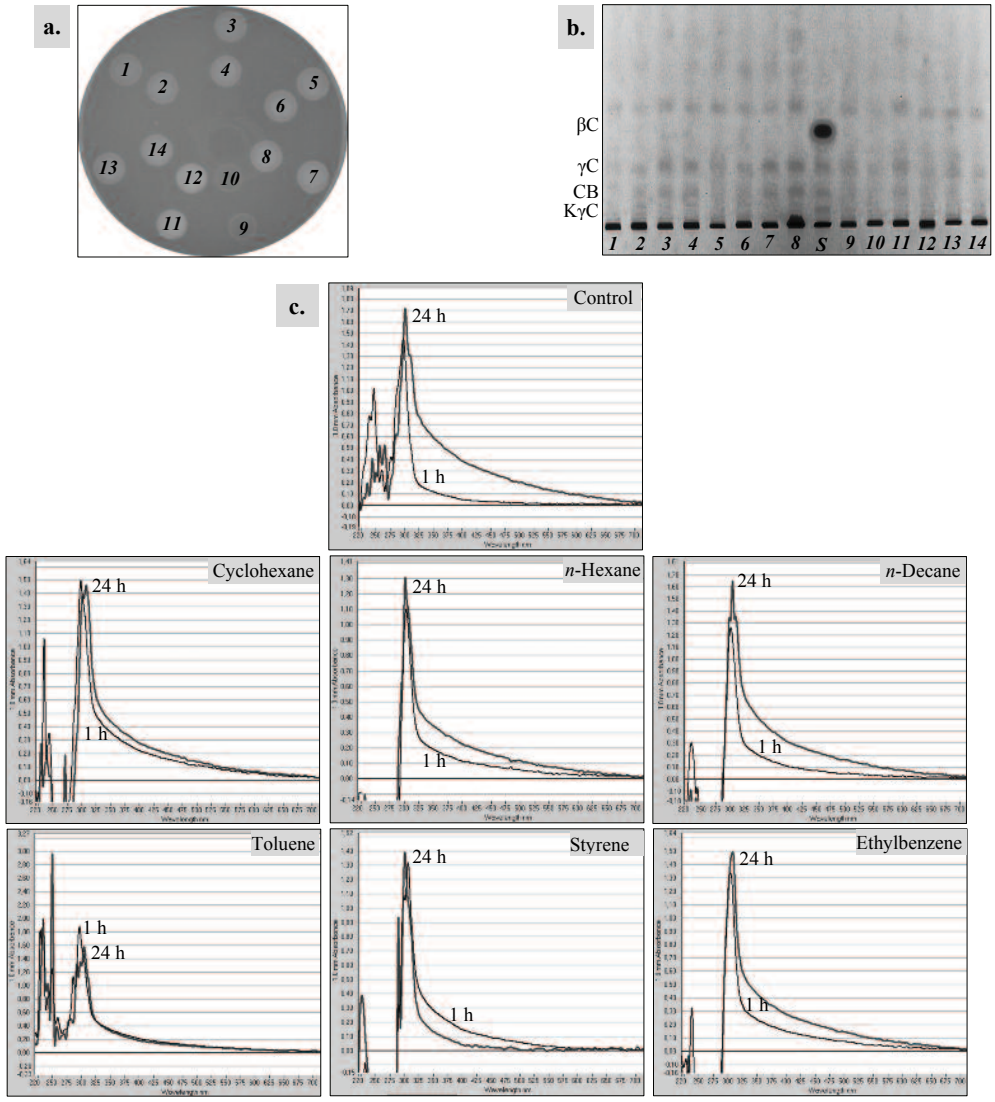

Figure 2. Pigments production by *Rhodococcus* sp. Po_4 after 1 % (v/v) organic solvents exposure
Bacterial cells cultivated 1 hour (spots or lanes ***1***, ***3***, ***5***, ***7***, ***9***, ***11***, ***13***) and 24 hours (spots or lanes ***2***, ***4***, ***6***, ***8***, ***10***, ***12***, ***14***) in LB-Mg medium, control (***1***, ***2***), cyclohexane (***3***, ***4***), *n*-hexane (***5***, ***6***), *n*-decane (***7***, ***8***), toluene (***9***, ***10***), styrene (***11***, ***12***), ethylbenzene (***13***, ***14***).
Panel a. The Petri plate was visualized under a 254 nm ultraviolet light.
Panel b. The TLC plate was visualized under a 254 nm ultraviolet light, carotenoids standard (S), 4-keto-γ-carotene (KγC), chlorobactene (CB), γ-carotene (γC), β-carotene (βC).
Panel c. UV/visible scanning spectra of the extracts were recorded between 220 and 700 nm.

On the TLC plate, no significant modifications in pigments profiles was observed in *Rhodococcus* sp. Po_4 cells 1 and 24 hours after alkanes and aromatics exposure, as compared with the controls. However, the spectral studies (Figure 2c) revealed the existence of some differences between carotenoid pigments extracted from *Rhodococcus* sp. Po_4 control cells and those extracted from cells exposed 1 and 24 hours to 1 % (v/v) alkanes and aromatics (especially toluene). According to literature (Godinho and Bhosle, 2008), the UV/visible absorption spectra of carotenoid pigments are of immense importance, since they aid a great deal in determining the structure of carotenoids. The UV/visible absorption scanning spectra of the pigment extract of *Rhodococcus* sp. Po_4 cells cultivated 1 and 24 hours on LB-Mg medium (control) showed peaks with absorption maxima at 290 nm and 300 nm, respectively. Different peaks with absorption maxima between 280 and 310 nm were detected in the pigment extract of *Rhodococcus* sp. Po_4 cells exposed 1 and 24 hours to 1 % (v/v) alkanes and aromatics.

Genomic fingerprinting. We showed previously (Stancu, 2012) that simultaneous exposure of *Vibrio alginolyticus* IBB_{Ct2} cells to salt stress and toxic organic solvents induced considerable modifications in their genomic fingerprinting 1 hour after solvent exposure, and these variations imply complex genomic rearrangements. Therefore, genomic DNA extracted from *Rhodococcus* sp. Po_4 control cells and those extracted from cells exposed 1 and 24 hours to 1 % (v/v) alkanes and aromatics were analyzed for their rep-PCR fingerprinting. Amplification using a gradient of annealing temperatures indicated optimal annealing temperatures of 40 °C for REP primers, 50 °C for BOXA primer (data not shown). BOXA primer produced the most complex amplified banding patterns for *Rhodococcus* sp. Po_4, with sizes ranging from 200 to 2,000 bp (Figure 3).

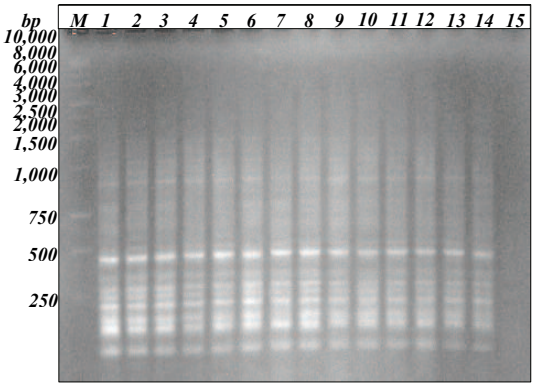

Figure 3. Genomic fingerprinting of *Rhodococcus* sp. Po_4 after 1 % (v/v) organic solvents exposure
Bacterial cells cultivated 1 hour (lanes ***1***, ***3***, ***5***, ***7***, ***9***, ***11***, ***13***) and 24 hours (lanes ***2***, ***4***, ***6***, ***8***, ***10***, ***12***, ***14***) in LB medium, control (***1***, ***2***), cyclohexane (***3***, ***4***), *n*-hexane (***5***, ***6***), *n*-decane (***7***, ***8***), toluene (***9***, ***10***), styrene (***11***, ***12***), ethylbenzene (***13***, ***14***), negative control DNA (***15***), 1 kb DNA ladder, Promega (***M***).

Exposure for 1 and 24 hours of *Rhodococcus* sp. Po_4 cells to alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) had no effect on their genomic fingerprinting, compared with control. Whole genome analysis of many *Rhodococcus* strains which have very large genomes indicates that they have acquired many gene copies, including the genes important for DNA-damage repair, in contrast to other bacteria with much smaller genomes but which are lacking DNA repairing functions (Larkin et al., 2006).

CONCLUSIONS

Rhodococcus sp. Po_4 showed good tolerance to both 1 % (v/v) alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene). Nevertheless, 1 % (v/v) alkanes were less toxic for *Rhodococcus* sp. Po_4 cells, compared with 1 % (v/v) aromatics.

The high organic solvent tolerance of *Rhodococcus* sp. Po_4 could be due to the presence in their large genome of some catabolic and trehalose-6-phosphate synthase genes. Additionally, *Rhodococcus* sp. Po_4 exhibits potential to synthesize carotenoid pigments which can improve the cell membrane impermeability to toxic organic solvents. By TLC analysis the same pigments profiles were revealed in *Rhodococcus* sp. Po_4 cells exposed or not to 1 % (v/v) alkanes and aromatics. However, the spectral studies revealed the existence of some differences between carotenoid pigments extracted from *Rhodococcus* sp. Po_4 control cells and those extracted from cells exposed to 1 % (v/v) alkanes and aromatics.

Exposure of *Rhodococcus* sp. Po_4 cells to 1 % (v/v) alkanes and aromatics had no effect on their genomic fingerprinting.

Due to its environmental origin and its metabolic potential, *Rhodococcus* sp. Po_4 could be used in the bioremediation of soil and water contaminated with different oily sludge.

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