Adaptation of *Rhodococcus* to Organic Solvents

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Contents

Abstract Bacterial tolerance and ability to adapt to organic solvents can be of valuable importance in biocatalytic and bioremediation processes. 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. In fact, these 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 such as benzene and toluene, and can aggregate and produce exopolymeric substances to protect the cell population from stressful environments.

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1 Introduction

Bacterial strains tolerant or adapted to organic solvents are of interest in both biocatalysis and bioremediation processes. These cells may work in the presence of organic solvents effective in overcoming the solubility of hydrophobic substrates and/or products in biocatalytic processes (de Carvalho and da Fonseca 2002a; Daugulis 2001; Heipieper et al. 2007), or biodegrade organic solvents released in the environment (Aislabie et al. 2006; de Carvalho and da Fonseca 2007; Leisinge 1996; Parales and Haddock 2004).

The first report of a bacterium tolerant to organic solvents was by Inoue and Horikoshi (1989), who discovered a *Pseudomonas putida* strain, IH-2000, able to tolerate and grow in the presence of 50% (v/v) toluene. This strain was, nonetheless, unable to metabolize toluene as sole carbon source. Since then, several Gram-positive strains belonging to the genus *Bacillus*, *Arthrobacter*, and *Rhodococcus* have shown high tolerance to organic solvents including benzene, which is much more toxic than toluene. *Rhodococcus* strains have been reported as efficient catalysts in the presence of organic solvents and also as degraders of these compounds because of their ability to metabolize a wide range of organic compounds under a wide set of conditions (de Carvalho and da Fonseca 2005a; Larkin et al. 2006). *Rhodococcus* cells are ubiquitous and are able to thrive in stressful environments, having been found in Antarctica (Bej et al. 2000; Pini et al. 2007), in the Artic (Thomassin-Lacroix et al. 2001; Whyte et al. 2002), at sea level (Bell et al. 1998), in the deep sea (Colquhoun et al. 1998), at high altitude (Margesin et al. 2003), and in semiarid soils (Pucci et al. 2000). The high hydrophobicity of *Rhodococcus* cells and the production of surface active compounds enhance their biocatalytic/degradation abilities.

1.1 Predicting Solvent Toxicity

The use of organic solvents in biocatalysis and bioremediation processes is limited as organic solvents can be extremely toxic to bacterial cells even at low concentrations. The toxicity of water-immiscible solvents may result from its direct contact to the cells (phase toxicity) and/or from the solvent molecules dissolved in the aqueous phase (molecular toxicity). These compounds partition to cell membranes, increasing its fluidity and disrupting the lipid bilayer, and ultimately cause cell death (Heipieper et al. 1991, 1994; Sikkema et al. 1995). In fact, organic solvents such as alcohols and phenols contained within natural oils and balsams have been widely used as antimicrobial agents since antiquity, long before their mode of action and effect was known.

Several authors have tried to present a way to predict solvent toxicity based on its physicochemical properties. In 1985, Brink and Tramper proposed that the suitability of a solvent to be used in a multiphasic biocatalytic system could be predicted by evaluating the solvent polarity and the molecular size of the solvent. The first could be determined by the Hildebrand solubility parameter, δ, while the

latter could be expressed as molecular weight or molar volume. In this system, high biocatalytic rates should be obtained using organic solvents with low polarity ($\delta < \sim 8$) and high molecular weight ($M > \sim 150$).

The antimicrobial action of a solvent was found later to better correlate to its hydrophobicity, measured as the logarithm of the octanol–water partition coefficient, log $P_{O/W}$, (Laane et al. 1987; Osborne et al. 1990; Sikkema et al. 1994). According to this scale, enzymes and microorganisms present a minimum of activity with solvents with log P values of 0–2 and 2–4, respectively, after which the use of solvents with increasing log P values will result in increased biocatalyst stability. However, the actual concentration of the solvent in the bacterial cell membrane will depend both on the solvent concentration in the water phase and on the partitioning of the solvent from the water phase to the membrane. In 1994, Sikkema and coworkers proposed the following equation to correlate the log $P_{O/W}$ value of a solvent and its partitioning value between the membrane and water, log $P_{M/W}$:

$$\log P_{M/W} = 0.97 \times \log P_{O/W} - 0.64. \quad (1)$$

Hydrophobic solvents, with log $P_{O/W} > 4$, accumulate in the membrane but will not reach a high membrane concentration and are not toxic because of their low water solubility. On the contrary, solvents with log $P_{O/W}$ between 1 and 4 present higher water solubility values, while being also able to partition to biological membranes, resulting in relatively high concentrations of these solvents in the membranes and high toxicity to the cells (de Bont 1998). The fact that solvents with high partition coefficient to the membrane, such as *n*-dodecane and *n*-hexadecane, are not toxic to bacterial cells is, apparently, contradictory. However, a "cut-off" in toxic effect around log P 4–5 was observed for microorganisms (Laane et al. 1985; Vermuë et al. 1993), above which the solvents do not present toxicity. The reasons presented involve low solubility of the solvents in the membrane bilayer or absence of a membrane disturbing effect, with significant responses being observed with different organisms and solvents (Sikkema et al. 1994). As pointed out by de Bont (1998), calculating the actual solvent concentration in membranes could be helpful. However, the author notes that several parameters that can also affect the partitioning of solvents, such as the composition of the biological membrane, are disregarded. Furthermore, many of the potentially interesting substrates are not very soluble in apolar solvents (those with higher log P values) and the reaction rates in favorable solvents are often low (Cassells and Halling 1990). Nevertheless, the log P values could be used as an indication of the biocatalyst behavior, even in processes requiring cofactor regeneration by viable cells (Fig. 1).

Recently, Hamada et al. (2008) compared several methods to predict bacterial predilection for organic solvents, namely: bacterial adhesion to hydrocarbon (BATH), contact angle measurement (CAM), hydrophobic interaction chromatography (HIC), and glass adhesion test (GAT). They concluded that CAM could be used to predict the dispersibility of bacteria in anhydrous organic solvents, while the BATH assays were better to predict the behavior of bacterial cells in organic–aqueous two phase systems. The differences in the obtained results using

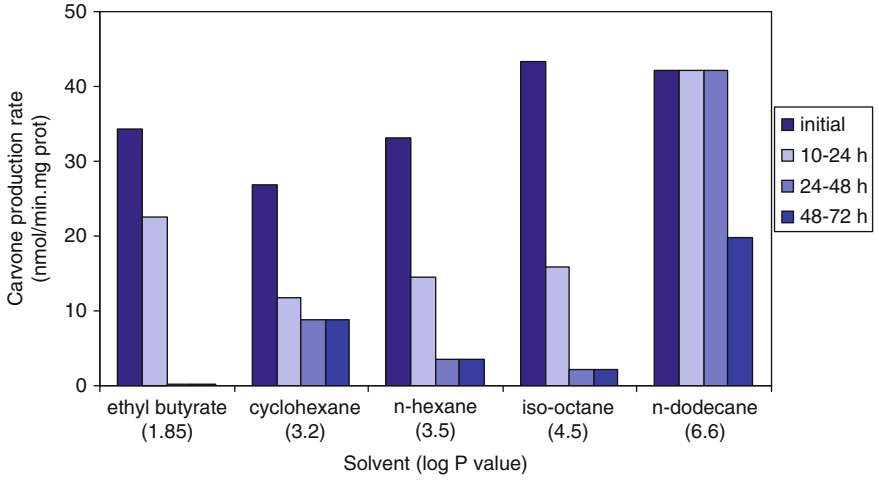

Fig. 1 Effect of the log *P* value of the solvent in *R. erythropolis* DCL14 cells carrying out the biotransformation of carveol into carvone in an organic-aqueous system (adapted from de Carvalho and da Fonseca 2002)

the two techniques were ascribed to the effect of electrostatic interactions between bacteria and oil droplets.

1.2 Effect of Solvents on Bacterial Cells

In a review on the cellular toxicity of lipophilic compounds, Sikkema et al. (1994) discussed the results published until then showing that the accumulation of lipophilic compounds occurs at various depths in the membrane bilayer, depending on the presence of hydroxyl, carboxyl, or phenyl groups. Hydrophobic cyclic hydrocarbons should accumulate in the acyl region of the membrane, while hydrophobic compounds should interact with hydrophobic end of acyl chains and more hydrophilic compounds should affect the hydration of the head groups of the membrane lipids. Small lipophilic compounds should intercalate with the acyl chains, resulting in membrane swelling and in an increased fluidity of the membrane. Since larger hydrophobic molecules affect both the inner and outer leaflet of the lipid bilayer, an increased ordering of the membrane lipids reduces the membrane permeability and fluidity but increases the bilayer width.

The cellular membrane of bacteria acts as a permeability barrier to solutes, regulating the intracellular environment, but is also responsible for the maintenance of the energy status of the cell, turgor pressure, signal transduction, and other energy-transduction processes (Sikkema et al. 1995). When solvents disrupt the membrane, loss of ions, metabolites, lipids and proteins, and impairment of the proton motive force across the membrane may occur, which stops the pH gradient

and the electrical potential and inhibits the function of membrane proteins. The ability of the microorganisms to maintain their biological functions under stressful conditions, such as the presence of organic solvents, results from changes in protein, sterol, hopanoid, and carotenoid content, and mainly changes in membrane lipid composition (Heipieper et al. 1994; Weber and de Bont 1996). The cells try to modify the fatty acid composition of the cellular membrane to maintain the membrane fluidity. The membrane fluidity is kept constant by changes in the degree of saturation of the fatty acids of the membrane phospholipids through a mechanism called "homeoviscous adaptation" (Sinensky 1974). Gram-negative bacteria, such as those of the genera *Pseudomonas* and *Vibrio*, isomerize unsaturated fatty acids from conformation *cis* to *trans* (Heipieper et al. 1992; Weber et al. 1994), which seems to be a special mechanism of adaptation to high concentrations of toxic compounds when de novo synthesis of lipids is not possible (Diefenbach et al. 1992). The studies carried out suggested that *cis* to *trans* conversion increases membrane ordering, decreasing the membrane fluidity. The level of isomerization has been found to correlate with the concentration (Diefenbach et al. 1992; Heipieper et al. 1992) and hydrophobicity (Heipieper et al. 2003) of the toxic compounds. Changes in the saturated–unsaturated and in the long chain–short chain fatty acid ratios can be seen as long-term changes to regulate membrane fluidity as synthesis of fatty acids is required. Other mechanisms involved in the response of Gram-negative bacteria to organic solvents include changes in the phospholipids headgroups, in the outer membrane proteins and lipopolysaccharides, and the action of efflux pumps. These mechanisms have been reviewed by Segura et al. (1999). Several other reviews have been published on solvent-tolerant bacteria (de Bont 1998; Isken and de Bont 1998; Ramos et al. 2002; Sardessai and Bhosle 2002; Sikkema et al. 1994). Although these works report solvent-tolerant *Rhodococcus* and other Gram-positive strains, the authors agree that without an outer membrane, Gram-positive bacteria should be less tolerant to organic solvents and an in-depth study on the mechanisms of solvent tolerance in these bacteria is still missing. The permeable cell wall of Gram-positive bacteria does not usually restrict the penetration of antimicrobial agents, but vancomycin-intermediate resistant *Staphylococcus aureus* strains are resistant due to a significantly thickened cell wall (Lambert 2002). Bacteria containing mycolic acids, such as mycobacteria and *Rhodococcus*, have cell walls with a high lipid content, which may act as a barrier to both hydrophobic and hydrophilic antimicrobials (Brennan and Nikaido 1995). In this chapter, the resistance and the mechanisms conferring adaptation capabilities to *Rhodococcus* strains will be presented and discussed.

2 Intrinsic Resistance to Organic Solvents

Some microorganisms have the ability to resist or tolerate concentrations of a certain compound that would kill or stress others. Intrinsic resistance is defined as an innate genetically controlled property of a bacterial cell that enables it to elude

the action of a biocide (Russell 1995). Resistance genes may reside on the chromosome, on a plasmid, or on a transposon. This natural resistance is the result of penetration barriers, metabolic pathways, or effective efflux pumps, while the acquired resistance results from an increased tolerance gained through a genetic change by which an organism and its progeny will be able to remain viable and/or multiply under the stress conditions. As long as the basal level of the repair systems is not surpassed by the stress damages, the cells will be able to survive.

Gram-negative bacteria are generally considered less susceptible to biocides than Gram-positive bacteria because their outer membrane acts as a permeability barrier. However, mycobacteria can present a high intrinsic tolerance as their cell is highly hydrophobic due to the mycoylarabinogalactan–peptidoglycan skeleton (McDonnell and Russell 1999). In the case of *staphylococci*, the cell wall is mainly composed of peptidoglycan and teichoic acid, which are not effective as penetration barriers. However, the physiological state of the cells can influence the susceptibility of the cells to biocides as the growth rate or growth under limiting nutrient conditions may change the thickness and degree of cross-linking of the peptidoglycan (Gilbert and Brown 1995). Slime producing *S. aureus* strains also present a higher resistance to antimicrobial agents than nonmucoid strains, indicating that exopolymeric substances may act as physical barrier or as absorbent of biocide molecules (McDonnell and Russell 1999). When the physical barriers allow the passage of the toxic compound, intrinsic resistance is given by efflux pumps that may extrude different types of compounds (Piddock 2006; Poole 2008).

In a study comparing the resistance of Gram-negative *P. putida*, and the gram-positive *Mycobacterium* sp. and *R. erythropolis* cells to various water miscible and immiscible solvents, it was found that of the three strains only *R. erythropolis* was able to endure high concentrations of water miscible solvents (de Carvalho et al. 2004). This result showed that *R. erythropolis* was able to maintain viability at higher solvent concentrations than *P. putida*, which should be more tolerant to organic solvents than Gram-positive bacteria because of the protective effect of the outer membrane (Isken and de Bont 1998). Fang et al. (2007) also found the Gram-positive *Bacillus subtilis* to be more tolerant than *P. putida* toward fullerene-based manufactured nanomaterials. *Bacillus*, *Rhodococcus*, and *Arthrobacter* have been found to be organic-solvent-tolerant even to benzene, one of the most toxic organic compounds (Sardessai and Bhosle 2002). The dominance of BTEX degrading Gram-positive bacteria in nearshore surface water and in sediments from the Pacific Ocean also contradicts the general idea that Gram-negative bacteria are the main group of organic pollutant degraders (Wang et al. 2008). Apparently, the more extensively linked peptidoglycan in Gram-positive cells (Sikkema et al. 1995) and the enzymes/substances excreted by these cells to emulsify/solubilize the organic solvents could play an important role in decreasing the solvent toxicity toward them (Abe et al. 1995).

Rhodococcus strains have been found to endure considerably high concentrations of known toxic compounds, for example, phenol, benzene, and toluene (Table 1). The work of Gutiérrez et al. (2003) with *Rhodococcus* sp. showed that

Table 1 Intrinsic resistance of several *Rhodococcus* strains to organic solvents

Strain	Compound	Tolerance	References
R. phenolicus G2P^T	Phenol	0.75%[a]	Rehfuss and Urban (2005)
R. opacus B-4, B-9, B-10	Benzene	90%[b]	Na et al. (2005)
R. opacus B-4	*n*-Tetradecane	100%	Hamada et al. (2008)
Rhodococcus sp. CN6,	*p*-Nitrophenol	100 mg/L[a]	Zhang et al. (2009)
R. rhodochrous S-2	*n*-Hexadecane	10%	Iwabuchi et al. (2000)
R. sp. NO14-1	*n*-Hexadecane	34%[c]	Margesin et al. (2005)
	Diesel oil	27%[c]	
	Phenol	12.5 mM[c]	
Rhodococcus sp. NO20-3	*n*-Hexadecane	54%[c]	Margesin et al. (2005)
	Diesel oil	37%[c]	
	Phenol	12.5 mM[c]	
Rhodococcus sp.	Benzene	200 mg/L	Gutiérrez et al. (2003)
R. equi 85F	Hydrogen peroxide	150 mM	Benoit et al. (2002)
R. erythropolis UPV-1	Phenol	1,000 mg/L	Prieto et al. (2002)
	Ethanol	40%	de Carvalho et al. (2004)
	Butanol	20%	
	Dimethylformamide	50%	
	Dodecane	5%	
	bis(2-Ethylhexyl) phthalate	5%	
	Toluene	1%[b]	
	Toluene	20%[d]	de Carvalho et al. (2007)
R. erythropolis DCL14	Methanol	15%[a]	de Carvalho et al. (2005)
	Ethanol	20%[a]	
	Butanol	2%[a]	
	Cyclohexanol	1%[a]	
	Dodecanol	5%[a]	
	Iso-octane	99.99%	de Carvalho et al. (2000)
	C5–C16 *n*-alkanes	0.25%[a]	de Carvalho and da Fonseca (2005b)
	Motor oils	2%[a]	
	Fuel oil	1.6%[a]	

[a]Organic solvent used as sole carbon source
[b]Growth observed
[c]At 10°C
[d]After 1 h incubation

although benzene caused an increase in membrane fluidity after 0.5 h, the cells did not change the fluidity of the membrane in the 6 h of the assay, during which the cells were alive. The authors ascribed this to a well-adapted inherent mechanism allowing the cells to survive benzene and other solvent "shocks" in the environment. Recently, Gutiérrez et al. (2009) studied the constitutive processes that confer resistance to benzene. Nonadapted *Rhodococcus* sp. 33 cells were able to endure shock concentrations of up to 1,000 mg/L of presolubilized benzene, a concentration usually lethal to most microorganisms. The production of an extracellular polymer and the composition of the cell wall and cell plasma membrane may be responsible for this high tolerance. In some cases, tolerance was related to the

capacity of the cells to degrade the toxic molecule and to use it as sole carbon and energy source (de Carvalho et al. 2005; de Carvalho and da Fonseca 2007). In the case of *R. rhodochrous*, the extracellular polysaccharide produced by the mucoidal strains was responsible for the tolerance of the cells to *n*-hexadecane (Iwabuchi et al. 2000).

In 1942, Withell observed an exponential relation between the duration of a stress episode and bacterial death, which could be explained by cells with different tolerance within the population. According to Booth (2002), survival to a stress agent largely occurs at the level of a single cell (as seen by the ability of a single cell to form a colony on an agar plate). After exposure to stressors that kill the majority of the cells, the heterogeneity of a population determines its survival as a small fraction of the cells may possess the necessary tools to endure the stress. The results obtained by de Carvalho et al. (2007) indicated that cells that are able to remain viable after the first seconds of exposure to high toluene concentrations will also be able to endure this toxic for longer periods. Two explanations could justify this result (1) the existence of toluene tolerant persistent cells within the population and (2) the tolerance is induced by cell exposure to a toxic, and until tolerance is acquired, cell death occurs at a toluene-concentration dependent rate.

3 Adaptation Mechanisms to Organic Solvents

Several reviews have been published on the tolerance of bacterial cells to organic solvents where the mechanisms of cell adaptation are discussed (de Bont 1998; Chapman 2003; Isken and de Bont 1998; Sardessai and Bhosle 2002; Segura et al. 1999; Sikkema et al. 1994). Among the most important mechanisms described are (1) changes in the cell membrane to modulate its fluidity, (2) the metabolism of the toxic compound or its inactivation, (3) increased efflux of the toxic compound. 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 nonpathogenic strains to nonantibiotic compounds are still scarce.

One basic idea observed in adaptation studies is that cells growing at a slow rate acquire general tolerance to the stress agent (Booth 2002 ; Sonnleitner 1998). Furthermore, by growing at slower growth rates, separate but overlapping pathways that confer tolerance of diverse stresses are induced, and the cells become simultaneously resistant to, for example, acid, heat, alkali, and hydrogen peroxide (Booth 2002). This mechanism of tolerance acquisition is particularly important in biofilms. The slow growth observed in the matrix-embedded cells and the limited transport of nutrients, metabolites, and oxygen between the surface and the interior of the biofilm could be responsible for an increased antibiotic and disinfectant resistance of biofilm cells over planktonic cells (Stewart and Costerton 2001; Donlan and Costerton 2002).

3.1 Adaptation of the Cell Wall and of the Cellular Membrane

Several papers have reported that when exposed to toxic organic compounds, tolerant bacterial strains change the fatty acid profile of their membrane (e.g., Heipieper et al. 1994; Isken and de Bont 1998; Sardessai and Bhosle 2002; Sikkema et al. 1995). The existence of an outer membrane allow Gram-negative bacteria to quickly modify and adapt the lipopolyssacharides, efflux pumps, and the fatty acid composition of the cellular membrane (Ramos et al. 2002). Among the modifications described are *cis*/*trans* isomerization of fatty acids, changes in the saturated/unsaturated fatty acid ratio, and changes in the phospholipids headgroups (de Bont 1998; Sardessai and Bhosle 2002; Segura et al. 1999). The *cis*/*trans* isomerization, which results in a lower penetration of the solvents through the inner membrane (Cronan 2002), is a short-term response, which takes place within 1 min after solvent exposure, while the remaining changes are long-term responses (Junker and Ramos 1999). The mechanisms involved in the adaptation of Gram-positive strains are not fully known (Fang et al. 2007; Nielsen et al. 2005), although it has been suggested that the mechanisms should be similar to those presented by Gram-negative bacteria (Ramos et al. 2002). In this case, the mycolic acids of *rhodococci* will act as a permeability barrier for hydrophilic compounds, and water-filled channels are required for their entrance in the cell. Lichtinger et al. (2000) identified and purified a channel-forming protein in *R. erythropolis* with a molecular mass of just 8.4 kDa with no significant homology to known protein sequences. The authors suggested that 2.0 nm channels were formed by protein oligomers in the cell wall, being highly cation selective due to negative charges located at the channel mouths.

n-Alkanes droplets have negative zeta potentials, for example, the value for *n*-hexadecane droplets is -46.0 ± -3.4 mV (de Carvalho et al. 2009). The negative zeta potential of *n*-alkane droplets is the result of selective adsorption of OH^- ions, which causes gathering of the excessive negative charge at the oil–water interface (Stachurski and Michalek 1996). Since *n*-alkanes and the channels at the cell wall have both localized negative charges, the entrance of these compounds could be prevented in the cells. However, several papers published showed that *Rhodococcus* cells are able to adhere directly to organic layers in organic–aqueous two-phase systems (de Carvalho and da Fonseca 2002b, 2003, 2007). Bouchez-Naïtali et al. (2001) also observed a direct uptake of *n*-hexadecane by four *R. equi* strains, which did not produce biosurfactants.

When comparing the effect of low and higher doses of buckminsterfullerene (*n*-C60) on *Bacillus subtilis* with the effect of hyperosmotic conditions, Fang et al. (2007) concluded that Gram-negative bacteria can present different responses to the same type of membrane-active compounds under different conditions, whereas Gram-positive bacteria can have the same response in terms of fatty acid composition under different stress conditions. *Rhodococcus* strains responded to the presence of organic solvents by changing the degree of saturation of the fatty acids of the cellular membrane, by changing the length of the fatty acids and mycolic acids according to the chain length of the carbon source, and by altering the percentage of

Table 2 Mechanisms of solvent tolerance observed at the cellular membrane level in *Rhodococcus* strains

Strain	Compound	Reported mechanism	References
Rhodococcus sp. 33	Benzene	Increased degree of saturation	Gutierrez et al. (1999)
Rhodococcus opacus GM-14, GM-29, and 1CP	Benzene, phenol, 4-chlorophenol, chlorobenzene, or toluene	Increase content of branched (10-methyl) fatty acids	Tsiko et al. (1999)
R. erythropolis DCL14	Short-chain alcohols (methanol, ethanol)	Decrease of degree of saturation	de Carvalho et al. (2005)
	n-alkanes and *n*-alkanols	Increase in degree of saturation	
R. erythropolis DCL14	C5–C16 *n*-alkanes	Saturated fatty acids with chain length corresponding to the substrate used; Net surface charge increased with chain length	de Carvalho et al. (2009)
R. erythropolis DCL14	Carveol and carvone	Lower percentage of long chain fatty acids; decrease of the unsaturation index	de Carvalho and de Fonseca (2007)
R. erythropolis DCL14	Toluene	C14:0 and C16:0 increased while C18:0 decreased; Increased percentage of iso-branched fatty acids when compared to straight-chain	de Carvalho et al. (2007)
R. erythropolis E1	C2–C7 *n*-alkanoic acid salts	MA profile according to the even–odd nature of the carbon chain of substrate	Sokolovská et al. (2003)
	C9–C15 *n*-alkanes	Changed cell wall permeability	
R. erythropolis 17, *Rhodococcus* sp. 20, *R. opacus*	Pentadecane, hexadecane	Fatty acids related to the chain length of the substrate	Alvarez (2003)
Rhodococcus sp. Q15	Alkanes at low T	Decrease of degree of saturation	Whyte et al. (1999)

branched fatty acids (Table 2). Rodgers et al. (2000), by using ^{13}C-enriched C16 and C18 alkanes and electrospray ionization fourier transform ion cyclotron resonance mass spectrometry, showed that complete mineralization was achieved by *R. rhodochrous*, with complete ^{13}C incorporation in the bacterial lipids. The incorporation of saturated fatty acids in the membrane phospholipids with chain length corresponding to the substrate used for cell growth has been found frequently. Studies carried out by Alvarez (2003) showed that most fatty acids in actinomycetes were related to the chain length of the substrate and also to β-oxidation derived fatty acids. Only when *R. erythropolis* DCL14 cells were grown on *n*-tridecane and

n-pentane, were the fatty acids C13:0 and C15:0 found in the cellular membrane, respectively (de Carvalho et al. 2009). Sokolovská et al. (2003) also observed two types of responses in mycolic acid patterns of *R. erythropolis* E1: lack of odd-numbered carbon chains when the cells grew on linear alkanes with even number of carbon atoms; mycolic acids with both even and odd carbon chains in cells grown on branched alkanes, or on mixtures of substrates. Furthermore, hydrocarbons can also be used in the biosynthesis of tryacylglycerols and wax esters under nitrogen starvation (Alvarez et al. 1996; Voss and Steinbüchel 2001).

Gutiérrez et al. (2003) compared a *Rhodococcus* sp. strain, able to tolerate and degrade high concentrations of benzene, with a benzene-sensitive mutant obtained by mutagenesis. The mutant was unable to increase the saturation degree of the fatty acids to the levels achieved by the wild type when exposed to benzene. The fluidity of the membranes increased after only 0.5 h of exposure to benzene, but the wild type was able to respond by changing the saturation:unsaturation ratio of the cellular membrane, especially by changing the proportion of myristic and oleic acids.

R. erythropolis cells adapted to 20–65% toluene concentrations by increasing the percentage of tetradecanoic and hexadecenoic acids while decreasing the percentage of octadecanoic acid (de Carvalho et al. 2007). The proportion of saturated iso-branched fatty acids also increased during toluene adaptation, while the amount of straight-chain fatty acids decreased. By increasing the content of iso-branched fatty acids, the cells decreased the fluidity or flexibility of the cellular membrane.

The content of branched (10-methyl) fatty acids also increased in *R. opacus* GM-14, GM-29, and 1CP, when the cells were grown on benzene, phenol, 4-chlorophenol, chlorobenzene, or toluene as sole carbon sources, as compared to fructose grown cells (Tsiko et al. 1999). A dose-related increase in the percentage of 10-methyl branched fatty acids was also observed as a response to increasing concentrations of phenol and toluene in strain GM-14, which is unable to metabolize toluene. 10-Methyl branched fatty acids are also present in *Rhodococcus koreensis* DNP505^T, which is able to degrade 2,4-dinitrophenol (Yoon et al. 2000). The role and position of 10-methyl branched fatty acids in *Rhodococcus* is still unclear but the results of Tsiko et al. (1999) suggest the cell envelope lipids that contain 10-methyl branched fatty acids should be involved in the adaptation of *Rhodococcus* strains to compounds affecting the cellular membrane, such as aromatics.

Changes in cell hydrophobicity may be promoted by using the cell response to organic compounds. By exposing *R. erythropolis* cells to the terpenes carveol and carvone in organic–aqueous systems, it was possible to demote biofilm formation and even to disrupt established biofilms (de Carvalho and da Fonseca 2007). Strain DCL14 responded to the presence of the solvents tested by decreasing the unsaturation index, which reflects the average number of double bonds per fatty acid chain, with increasing number of carbons in the alkane chain. In the presence of carveol or carvone, the cells increased the unsaturated index, thus counteracting the effect of the solvents. The presence of polyunsaturated fatty acids has been reported to allow the cells to change membrane fluidity (Melchior 1982) and permeability (Russell

1988). The major response to the presence of these two terpenes was, however, a decrease in the percentage of fatty acids with a number of carbons higher than 16 (de Carvalho and da Fonseca 2007). Since a direct relation between these fatty acids and cell hydrophobicity was found, by decreasing the percentage of fatty acids with more than 16 carbon atoms, cell hydrophobicity decreased, which resulted in the dispersion of cells previously aggregated because of the presence of organic solvents.

The cell surface hydrophobicity of *Rhodococcus* sp. Q15, which is able to mineralize alkanes, diesel, and Bunker C crude oil at both 5°C and 24°C, was higher after growth on diesel fuel and hexadecane than when the cells were grown on glucose-acetate (Whyte et al. 1999). The carbon source strongly influenced the fatty acid profile of the cells, with small amounts of C18 fatty acids and greater amounts of C16 and C14:0 fatty acids being observed in cells grown on *n*-hexadecane when compared to those grown on glucose-acetate. The degree of saturation of the fatty acids of the membrane of strain Q15 decreased as response to a lower growth temperature: the membrane contained relatively saturated fatty acids at 24°C and relatively unsaturated fatty acids at 5°C. This happened independently of the substrate used, although the decrease in the degree of saturation occurred at a lesser extent when the cells grew on hydrocarbons than on glucose-acetate. The cells were thus able to modulate the membrane fluidity to respond to both the influences of low temperature and hydrocarbon toxicity.

The most interesting physicochemical surface properties adaptation of *Rhodococcus* cells to organic solvents was observed with *R. erythropolis* growing on C5–C16 *n*-alkanes (de Carvalho et al. 2009). A strong correlation between the *n*-alkane chain length and the zeta potential of the bacterial cells was observed, with the cells even becoming positive when they grew on C14–C16. Most known bacteria only exhibit negative surface charges at circum neutral pH (Jucker et al. 1996) and the fact that *R. erythropolis* DCL14 presents a positive surface charge is quite remarkable. According to the extended Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory of colloidal stability (Van Oss 1995), in which electrostatic repulsion, van der Waals attraction, and acid–base (hydrophobic) interactions are considered, the adhesion capacity of bacterial cells is inversely correlated with the (negative) surface charge of the cell. Since most natural surfaces are negative, adhesion will only take place when the electrostatic repulsion is overcome by attractive forces (e.g., van der Waals, hydrophobic interactions) between the bacteria and the surface. In the case of strain DCL14, the positive surface charge will contribute to the attachment of the cells to negatively charged surfaces such as *n*-hexadecane droplets (zeta potential of -46.0 ± -3.4 mM).

3.2 Biocatalysis and Biodegradation of the Toxic Compound

Bioconversion or mineralization of a toxic compound has been presented as a mechanism for bacterial strains to thrive in its presence. However, although some studies indicate that tolerance to solvents derives from the capacity of the cells

to metabolize them, other works suggest that conversion or metabolism of organic solvents is not essential to tolerance. The degradation may mediate the resistance of some bacterial strains to solvents but it cannot be the main mechanism conferring tolerance to a broad number of solvents (Isken and de Bont 1998).

Rhodococci present a broad catabolic diversity and enzymatic capabilities, increasing their importance in environmental and biotechnological processes (Bell et al. 1998; de Carvalho and da Fonseca 2005a; Martínková et al. 2009; Warhurst and Fewson 1994). Their exceptional ability to resist and degrade hydrophobic compounds and xenobiotics is related to the presence and mobilization of large linear and circular plasmids, while the presence of multiple pathways and gene homologous enhance their versatility (Larkin et al. 2005, 2006; van der Geize and Dijkhuizen 2004). Works reporting the ability of *Rhodococcus* strains to act as whole-cell biocatalyst even in anhydrous organic solvents have also been published (e.g., Yamashita et al. 2007).

In a work carried out to study the effects of organic solvents in organic–aqueous systems on *R. erythropolis*, *Xanthobacter* Py2, *Arthrobacter simplex*, and *Mycobacterium* sp. NRRL B-3805, principal components analysis was used to interpret the data (de Carvalho and da Fonseca 2004). The variables used to construct the data matrix were cell viability, cell morphological parameters (e.g., size, elongation factor, circularity), number of cells in clusters, and the conditions to which the cells were exposed [substrate concentration, carbon source used for growth, adaptation time to the solvent prior to substrate addition, and physical properties of the solvents (e.g., density, molecular weight and log *P*)]. Over a third of the variability of the data related to *R. erythropolis* could be explained by solvent toxicity. When studying the effect of several solvents, present at different organic:aqueous ratios, on whole cells of *R. erthropolis* DCL14 carrying out the biotransformation of (−)-carveol to (−)-carvone, principal components analysis showed that 41.2% of the variance of the data responsible for the cell behavior could be ascribed also to solvent toxicity (de Carvalho et al. 2003).

In two-phase systems, *R. erythropolis* cells migrate toward the organic phase because of their high cell hydrophobicity (de Carvalho and da Fonseca 2002a, b, 2003). When emulsion samples were collected and droplets of solvent were observed by fluorescence microscopy, it was found that the cells partitioned between the organic and the aqueous phase. Part of the cell population was even inside the solvent droplets (Fig. 2a, b). The images with cells on the organic phase were not just the result of a superposition of different planes, as shown by a technique developed by de Carvalho and da Fonseca in 2003 (Fig. 2c), which allows the observation of 3D solvent droplets adsorbed to solid particles, for example, of silica gel. Cells were preferentially inside the organic droplets in the solvents with high log *P* value, being the percentage of cells in droplets lower when the organic phase was more toxic (Table 3). However, in the presence of cyclohexane, which has a log *P* value of 3.2 and should be toxic, most of the cells were also positioned inside the solvent droplets. Cells directly positioned in the organic phase can access the dissolved hydrophobic substrates in biocatalytic processes (de Carvalho et al. 2000; de Carvalho and da Fonseca 2002b), and can degrade organic

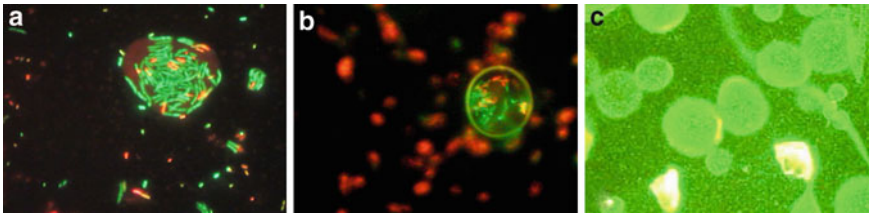

Fig. 2 *R. erythropolis* cells inside *n*-dodecane droplets. (**a**, **b**) solvent droplets trapped between a slide and cover slip (horizontal and vertical field widths equal to 0.16 and 0.12 mm, respectively); (**c**) solvent droplets adsorbed on silica gel particles (horizontal and vertical field widths equal to 0.4 and 0.3 mm, respectively)

Table 3 Percentage of *R. erythropolis* cells inside solvent droplets with different toxicities at different phase ratios

Phase ratio	0.0005					0.0025					0.025		
log *P* (solvent)	1.85	3.2	4.5	6.6	−1.35	1.85	3.2	3.5	9.6	12.88	−1.35	6.6	12.88
Cells in droplets (%)	64.9	93.5	72.5	97.7	73.4	58.5	99.0	57.2	73.0	99.3	68.0	91.7	97.6

The cells were carrying out the conversion of carveol into carvone in the organic–aqueous two-phase systems

solvents by direct uptake in bioremediation systems (Bouchez-Naïtali et al. 2001; de Carvalho and da Fonseca 2007). In these cases, the growth and conversion rates obtained are independent of the interfacial area, due to the strong adsorption of the bacterial cells at the solvent–aqueous interface.

In a paper regarding adaptation of microbes, Sonnleitner (1998) discussed the role of static effectors (such as concentrations of substrates and products) that affect the system instantaneously and independently of time (limiting or inhibiting the cells) and dynamic effectors that are time dependent and associated to changes in the physiological state or protein synthesis or degradation. When in contact with a toxic compound, the cells may express an enzyme(s) or use an alternative pathway to catabolize it and decrease the concentration of the biocide in the microenvironment surrounding the cells. For economical reasons, the cells usually only express the required enzymes after being exposed to the toxic compound.

By slowly increasing the concentration of solvent, substrate, and product, it was possible to adapt *R. erythropolis* DCL14 cells in an air-driven direct contact bioreactor (de Carvalho and da Fonseca 2002b). By recirculating *n*-dodecane through the column reactor at a rate slow enough so that the biotransformation could only take place at a small extent, the cells were able to adapt. When the biotransformation was performed after the adaptation period, the product carvone reached a concentration of 94 mM after 310 h of operation, thus overcoming carvone inhibition (observed at carvone concentrations of 50 mM). By increasing the adaptation period to 136 h, carvone accumulation reached 259 mM. Since the biomass was kept relatively constant during the experiments, the adapted cells were able to produce much more product than the nonadapted cells.

A similar strategy could be used to adapt strain DCL14 cells to toluene to allow its degradation at high concentrations in *n*-dodecane–aqueous systems (de Carvalho et al. 2007). Only 10.5% of the initially nonadapted cells remained viable after 1 h exposure to 20% (v/v) toluene. Cell adaptation was carried out by adding a toluene pulse, whenever its concentration reached ca. 50% of the initial value, to double the previous initial concentration (i.e., if the initial concentration was 1%, a pulse of toluene was added when toluene concentration reached 0.5% so that the concentration would be 2%). Curiously, toluene degradation rate increased with the increasing toluene concentrations added to the reactor. By using this strategy, the cells could endure a maximum toluene concentration of 4.9 M, which corresponds to 52.4% (v/v) in the organic phase, toluene being consumed at 10.7 mg/(h.mg protein). Once more, the amount of biomass was kept nearly constant through out the 35 days of the experiment. The fatty acid composition of the adapted cells presented a higher amount of branched fatty acids and ca. 40% decrease in the amount of straight-chain fatty acids. Interestingly, cells adapted to toluene presented a significant increased resistance to ethanol, silver ions, and iodine when compared to nonadapted cells.

The results obtained with *R. erythropolis* DCL14 suggest that toluene metabolism should be related to toluene tolerance mechanisms, as a correlation between toluene consumption rate and initial toluene concentration was found. The presence and cometabolism of *o*-, *m*-, and *p*-xylene further enhanced the degradation of toluene (de Carvalho et al. 2007). Leneva et al. (2009) could also adapt *R. opacus* 412 and *R. rhodnii* 135 to phenanthrene and anthracene on solid medium. The cells accelerated the metabolism and became able to grow on phenanthrene as sole carbon and energy source in liquid medium. *R. erythropolis* CCM 2595, although able to use phenol, pyrocatechol, resorcinol, *p*-nitrophenol, *p*-chlorophenol, hydroquinone, and hydroxybenzoate, was strongly affected by the substrate and initial concentration, and while some of the monoaromatic compounds suppressed the ability of strain CCM 2595 to use phenol in binary mixtures, others were strong inducers of phenol 2-monooxygenase (Čejková et al. 2005). In the latter case, the mixtures were more rapidly metabolized, if the cells were preexposed to the substrates. Cold-adapted *Rhodococcus* sp. strain NO14-1 and strain NO20-3 were able to fully degrade 12.5 mM phenol after 25 and 28 days, respectively, but when exposed to 15 mM phenol only 4 mM were degraded after 10 days and no further degradation was observed over 36 days (Margesin et al. 2005). Adaptation of *R. rhodochrous* 172 on agar mineral medium with fluorene for 6 months resulted in rapid growth without lag phase of the adapted cells: complete degradation of 12 mg/L fluorene was achieved in liquid medium within 5 days, while the nonadapted cells were unable to grow (Rubashko et al. 2006). The results presented are an indication that in these strains tolerance and degradation capacity may be strongly related.

The initial results obtained by Na et al. (2005) also seemed to indicate a crucial role between solvent tolerance and its utilization or degradation in *R. opacus* B-4. However, a mutant defective in benzene dioxygenase was as tolerant to organic solvents as the wild strain B-4. This suggests that conversion or degradation is

not essential for organic solvent tolerance of *R. opacus* B-4. Two mutants of *Rhodococcus* sp. 33 unable to degrade benzene were still tolerant to 500–800 mg/L of benzene (nonadapted strain 33 cells were able to tolerate ca. 1,000 mg/L), also confirming that benzene-degradation is of minor importance to the tolerance of these variants (Gutiérrez et al. 2009). Mosqueda et al. (1999) also suggested that toluene metabolism is not involved in toluene tolerance in *P. putida* DOT-T1. Independently of the mechanisms used by each strain, solvent tolerance is important as it allows bacterial growth at high organic solvent concentrations, which is of paramount importance in the bioremediation of sites contaminated with compounds such as benzene and toluene (Chen et al. 2009; Na et al. 2005).

Lately, organic solvent tolerant strains have received a further notice because biodesulfurization of petroleum occurs in the presence of high concentrations of hydrocarbons. Many of the described competent bacteria to perform desulfurizations are *R. erythropolis* strains, for example, IGTS8, N1-43, D-1, and KA2-5-1, with strain IGTS8 being the best characterized. The *dszA*, *B*, and *C* genes primarily responsible for DBT metabolism are located in a single operon on a large plasmid in strain IGTS8 and in other related strains (Monticello 2000). Five strains, able to utilize dibenzothiophene (DBT) as sole sulfur source and convert it to hydroxybiphenyl (HBP), isolated from coal storage sites in the north of France and from soils contaminated with heavy crude oil with high sulfur content belonged to the *Rhodococcus/Gordonia* cluster (Abbad-Andaloussi et al. 2003). All strains were able to use DBT in 95% *n*-hexadecane, used as model for diesel oil, although no activity was observed at *n*-hexadecane concentration of 99%. Some of the strains used by Bouchez-Naïtali et al. (2004), namely *Rhodococcus* sp. MK7C1 and MK2.4, exhibited good resistance to solvents, being even more tolerant than nondesulfurizing *Pseudomonas* strains, which could explain why transference of biodesulfurizing genes into Gram-negative strains did not promote biodesulfurization activity. An increased DBT desulfurization activity could be increased in biphasic systems, as reported by Ohshiro et al. (1995) with *R. erythropolis* H-2 in 70% *n*-tetradecane, Patel et al. (1997) with *R. erythropolis* IGTS8 in 50% *n*-hexadecane, and Abbad-Andaloussi et al. (2003) with *Rhodococcus* sp. strain I-2207 in the presence of 50% *n*-hexadecane. Nevertheless, total biodesulfurization of fossil fuel at industrial scale is not expected to occur in the near future (Soleimani et al. 2007).

3.3 Other Mechanisms of Protection

Iwabuchi et al. (2000) reported an association between colony morphotypes and oil tolerance in *R. rhodochrous*. The mucoidal strain was resistant to 10% (v/v) *n*-hexadecane while the rough derivatives were sensitive to this concentration. Furthermore, when the extracellular substance (EPS) produced by the mucoidal strain was added to cultures of the rough strain, the latter acquired resistance to *n*-hexadecane. Rough strains are hydrophobic and mucoidal strains are hydrophilic.

The EPS produced could confer tolerance to organic solvents by lowering the surface hydrophobicity, since, as Kobayashi et al. (1999) showed, low cell surface hydrophobicity could act as a defense mechanism against these compounds. The EPS produced by *R. rhodochrous* S-2 was even effective in stimulating the degradation of aromatic compounds in crude oil by native marine bacteria (Iwabuchi et al. 2002). Urai et al. (2007) also suggested that the large quantity of extracellular polysaccharides produced by *R. erythropolis* PR4 cells (able to degrade several hydrocarbons, including pristine) play an important role in hydrocarbon tolerance.

Cells of *R. erythropolis* DCL14 also present a non-EPS and a EPS producer variant. When exposed to the terpenes carveol and carvone and to organic solvents, the degree of saturation of the membrane phospholipids decreased, while the reverse was observed on the EPS producer counterpart (de Carvalho and da Fonseca 2007). The presence of EPS altered the level of cell exposure to solvents and terpenes. In the presence of organic solvents, part of the initially rough DCL14 population may start producing EPS (de Carvalho and da Fonseca 2002b, 2007). When these cells were under organic solvent stress-induced conditions, the small part of the population that remained viable produced colonies with a different phenotype: they were yellow or white while the nonstressed cells produced pink colonies (de Carvalho et al. 2004). Furthermore, under conditions that allow high cell viability, cells that died presented no significant morphological changes when compared to viable cells. However, under aggressive conditions, nonviable cells were much larger, probably because of an increase in membrane fluidity, with the viable cells succeeding in decreasing their surface area to minimize the area of contact with the toxic agent (de Carvalho and da Fonseca 2004; de Carvalho et al. 2004).

Several rhodococci strains have been reported to produce biosurfactants, usually glycolipids, such as *R. erythropolis* 51 T7 (Marqués et al. 2009), *R. erythropolis* DSM 43215 (Lang and Philp 1998) and *R. equi* Ou2 (Bouchez-Naïtali and Vandecasteele 2008). A relation between the surface tension of the culture medium and the alkane chain length during C5–C16 growth of *R. erythropolis* DCL14 indicated the production of a biosurfactant in the presence of *n*-alkanes with longer carbon chains (de Carvalho et al. 2009). The significant reduction of the surface tension to values lower than 30 mN/m indicates the biosurfactant power of the compound produced.

A common pattern for growth on long-chain alkanes in bacteria not producing biosurfactants is the formation of cellular flocs (Bouchez-Naïtali et al. 2001). In this case, the degradation rates during linear growth do not increase with interfacial area but with the efficiency of stirring, the interfacial uptake being limited by floc formation. Cell clustering was also visible with *R. erythropolis* DCL14 cells growing on *n*-dodecane, *n*-tetradecane, and *n*-hexadecane (de Carvalho and da Fonseca 2005b; de Carvalho et al. 2009). Biofilm formation can also been seen as a form of protection of cells to stress environments as cells inside the biofilm matrix are more protected (Heipieper et al. 1991). For example, no toxic effect of *n*-octane on biofilm growth was observed while the cells in suspension were strongly

inhibited by the accumulation of 1-octanol, a metabolite of the alkane monooxygenase during growth on *n*-octane (de Carvalho et al. 2009).

The action of efflux pumps responsible for the efflux of organic solvents from the inside of cells could also be responsible for adaptation of *Rhodococcus* cells to these compounds. Several antibiotic efflux pumps have been reported in Gram-positive strains, especially in *S. aureus*. However, studies on solvent efflux pumps in these bacteria are nearly inexistent (Fernandes et al. 2003).

4 Application

A large set of enzymes from rhodococci cells have been reported and identified, allowing these cells to carry out a large array of bioconversions and degradations: from oxidations to dehydrogenations, epoxidations, hydrolysis, hydroxylations, dehalogenations, and desulfurizations. Since the cells are able to tolerate and adapt to organic solvents, bioreactions can be done in nonconventional media, using low water soluble substrates, which makes this genus one of the most promising in biotechnology.

The ability of *Rhodococcus* cells to metabolize hydrocarbons (even at significantly high concentrations and under a wide variety of environments), to produce biosurfactants (required to increase the bioavailability of low water soluble organic solvents) and to change the physicochemical properties of the cellular surface (making these cells highly adaptive) make these cells ideal candidates to in situ bioremediation of hydrocarbon contaminated sites.

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