Title: Comparative benzene-induced fatty acid changes in a *Rhodococcus* species and its benzene-sensitive mutant: the possible role of myristic and oleic acids in providing tolerance

Authors: Tony Gutiérrez,[*] Robert P. Learmonth,[§] Peter D. Nichols,[#] and Iain Couperwhite[*]

[*] School of Microbiology and Immunology, The University of New South Wales, Sydney, N.S.W. 2052, Australia;

[§] Department of Biological and Physical Sciences, and Center for Rural and Environmental Biotechnology, University of Southern Queensland, Toowoomba QLD 4350, Australia;

[#] CSIRO Marine Research, GPO Box 1538, Hobart, Tasmania 7000, Australia.

Author to whom correspondence should be addressed:

Dr. Tony Gutierrez, Email tony-gutierrez@sams.ac.uk

Abstract

A Gram positive bacterium of the genus *Rhodococcus* was isolated from a contaminated site in Sydney, Australia, for its ability to tolerate and degrade high concentrations of benzene. To identify fatty acids that may impart this *Rhodococcus* sp. with tolerance to toxic solvents, a benzene-sensitive strain, labeled M2b, was isolated from this organism using EMS mutagenesis. A comparative analysis of fatty acid profiles showed that strain M2b was unable to increase its saturated:unsaturated ratio of fatty acids to the level achieved by the *w-t* strain when both strains were challenged with benzene. This was found to be due to M2b's increased abundance of myristic acid, and decreased abundance of oleic acid. In addition, by measuring the generalized polarization of the fluorescent membrane probe laurdan using fluorescence spectroscopy, we have shown for the first time the effects of an aromatic hydrocarbon on the membrane fluidity of a *Rhodococcus* sp. The fluidity of the membranes increased within only 0.5 hours of exposure to benzene, thus suggesting the partitioning of benzene within the lipid bilayer. The response of this *Rhodococcus* sp. to benzene may suggest a mechanism to how other microorganisms survive when toxic solvents are released within the vicinity of their environment.

Keywords: benzene, membrane fluidity bioremediation, fatty acids, myristic acid, oleic acid, *Rhodococcus*, cell membrane, solvent tolerance.

INTRODUCTION

Aromatic compounds are present in natural ecosystems as common components of many secondary plant metabolites and as products released from the partial decomposition of lignin and protein (Claus and Walker, 1964; Middlehoven *et al.*, 1992). Due to human intervention, however, many aromatic solvents have been introduced into the environment in high concentrations, often via crude oil spills and leaky underground petroleum tanks. Benzene, a derivative of crude oil, is one of the most toxic environmental pollutants recognized. It has been ranked as the fifth most hazardous pollutant based on a total of 466 different volatile organic compounds found at 888 contaminated sites in the United States and other countries (Siegrist, 1992). Previously, our group isolated a Gram positive bacterium from a contaminated site near a chemical plant in Sydney, Australia, based on its ability to tolerate and efficiently degrade concentrations of benzene reaching its maximal solubility (1800 mg/L) in aqueous media (Paje *et al.*, 1997). The organism was partially characterized and found to belong to the *Rhodococcus* genus. Like the pseudomonads, many species within this genus have been recognized to tolerate and degrade various pollutants. They have been described as very robust and found inhabiting a wide range of environments (Warhurst and Fewson, 1994).

The isolation of solvent-tolerant microorganisms has opened a new era of investigation directed towards elucidating the mechanisms that confer solvent-resistance. This should help to further our understanding into how they are able to exist in environments that are ridden with toxic chemicals where most other microorganisms would not survive. Numerous reports in the literature, mainly from the past two decades, have shown that various hydrocarbon-tolerant microorganisms produce qualitative and

quantitative changes in their membrane fatty acids in response to toxic organic compounds (Heipieper *et al.*, 1994; Sikkema *et al.*, 1995; Weber and de Bont, 1996). Such changes have been shown to decrease the fluidity of cell membranes. This has been proposed to give them greater structural rigidity and be a mechanism to tolerate the organic solvents (Sikkema *et al.*, 1995). To-date, only three reports exist that have studied the effects of aromatic hydrocarbons on the fatty acid composition in any one *Rhodococcus* spp. (Hamilton *et al.*, 1995; Tsitko *et al.*, 1999; Gutierrez *et al.*, 1999). We have shown that our *Rhodococcus* increases its ratio of saturated:unsaturated cellular fatty acids during growth with benzene (Gutierrez *et al.*, 1999). Such changes are often associated with decreasing the fluidity of cell membranes, and are suggested to reduce the toxicity of solvents like benzene . Although changes in the abundance of various fatty acids have been shown to lead to the fluctuations observed in the sat:unsat ratio, the specific fatty acids that play a contributing role in the tolerance of this organism to toxic solvents in the environment remain to be elucidated.

The present study was conducted to identify fatty acids in a *Rhodococcus* sp. that may be involved in its adaptive response associated with tolerating exposure to benzene. For this, a benzene-sensitive mutant, M2b, was isolated and its fatty acid profiles determined and compared to those of the parent strain (*w*-*t*) when both strains were exposed to benzene. We also report for the first time the effects of an aromatic hydrocarbon on the cell membrane fluidity of a *Rhodococcus* sp.

METHODS AND MATERIALS

Organism and growth conditions

A highly benzene-tolerant *Rhodococcus* sp., previously isolated from a contaminated site near Port Botany in Sydney, Australia (Paje *et al.*, 1997), was used. A benzene-sensitive mutant, labeled strain M2b, was isolated from this *Rhodococcus* (*w-t*) as described below. Stock cultures were maintained at 4°C on Luria-Bertani (LB) agar medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar per liter of water). Inocula were prepared in 50 mL of minimal medium (MM), with colonies from a fresh plate, and supplemented with mannitol (1%) and/or benzene (supplied via the vapor phase) as required (Paje *et al.*, 1997). Cells were grown to the mid-exponential phase at 28 °C and 190 rpm prior to use as inocula at a final level of 4% (v/v).

Isolation of benzene-sensitive mutants

The method for generating benzene-sensitive mutants from the *w-t* was performed using chemical mutagenesis with ethyl methanosulfonate (EMS), as adapted from the method by Eisenstadt *et al.* (1994). This involved subjecting the cells to a 4% solution of freshly prepared EMS (Sigma), pre-warmed to 37 °C, for 2 hours. The cells were then washed twice with 0.1M phosphate buffer. To select for non-growing benzene-sensitive mutants, the cells were re-suspended in a 50-mL volume of LB broth supplemented with 0.2 mg/L of pre-solubilized benzene and 2 mg/L of ampicillin. After 4 hours of incubation (28 °C, 190 rpm), the cells were harvested and appropriate dilutions performed for replica spread-plating on LB agar medium. The plates were incubated at room temperature in a cotton wool-plugged desiccator for 72 hours with benzene supplied via the vapor phase. For a comparative selection of sensitivity to the benzene,

duplicates of these plates were maintained under identical conditions without benzene. Colonies of less than 0.5 mm in diameter were selected as benzene-sensitive mutants from plates incubated in the desiccator with the benzene.

Effects of benzene on the fatty acid profiles of strain M2b

To identify fatty acids involved in conferring the *w-t* with a tolerance to benzene, the total lipid profiles of the benzene-sensitive mutant, strain M2b, were compared to those previously published for cells of the *w-t* grown under identical conditions (Gutierrez *et al.*, 1999). For this, three cotton wool-plugged 250-mL Erlenmyer flasks containing 50 mL of MM were used. Culture 1 contained mannitol, culture 2 contained benzene, and culture 3 both mannitol and benzene, as carbon sources. Each was inoculated with the 4% inoculum and incubated (28 °C; 190 rpm) until a cell turbidity (OD_{600}) of approximately 0.4 (10^{-1} dilution) was reached. The cells were then harvested prior to use for lipid extraction and fatty acid analysis.

For lipid extraction, whole cell lysates were prepared of wet pellet (triplicate samples, 400 mg) by saponification in a solution of 13% NaOH in 50% (v/v) aqueous MeOH at 100°C for 30 min. The fatty acids were then methylated in a solution of 3.25M HCl in aqueous methanol at 80°C for 10 min. The fatty acid methyl esters (FAME) were extracted with hexane/methyl tertiary butyl ether (MTBE) and base washed in a dilute solution of 1.2% NaOH. Triplicate 0.1 mL samples of the hydrophobic phase, constituting the fatty acid fraction in hexane and MTBE, were then dispensed into 1 mL glass vials and hermetically capped with Teflon-lined rubber septa for gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis.

Benzene's effect on the membrane fluidity of the w-t

Two types of culture treatments were prepared to determine benzene's short-term effect on the cell membrane fluidity of the *w-t*. These were performed in hermetically closed screw-capped 250-mL Teflon tubes containing 100 mL of MM and inoculated with a washed 50-mL suspension of benzene-unexposed cells in the exponential phase. One culture contained 1% (v/v) mannitol (no benzene), and the other contained pre-solubilized benzene (200 mg/L) plus the 1% mannitol. Samples for fluorescence analysis were taken at the point of inoculation and periodically thereafter.

To determine benzene's long-term effect on the membrane fluidity of the *w-t*, three types of culture treatments were prepared - one with mannitol, the second with benzene, and the third with both mannitol and benzene as carbon sources. Each was inoculated with the 4% inoculum (benzene-unexposed cells) and grown to the exponential phase prior to sampling for spectrofluorometric analysis.

Membrane fluidity was assessed by measurement of generalized polarization (GP) using the fluorescent membrane probe laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) as developed for application with microorganisms (Parasassi *et al.*, 1990; Learmonth and Gratton 2002). When necessary, cell cultures were diluted to OD_{600} = 0.3 (10^{-1} dilution) for fluorescent probe labeling. Five µL of laurdan (Molecular Probes, Eugene, OR), from a 1 mM stock solution in ethanol, was added to 5 mL of cell culture. This was incubated for 30 min at room temperature in the dark prior to spectrofluorometric analysis.

Analytical

GC analysis was performed with a Hewlett Packard 5890A GC as described elsewhere (Gutierrez *et al.*, 1999). Peaks were quantified with DAPA and/or Waters Millennium Scientific software. Individual components were identified using GC-MS data (see below) and comparing retention time data with those obtained for authentic and laboratory standards. The standard deviations (SD) were ≤ 5% of the mean value determined for each individual fatty acid.

GC-MS analyses were performed on a Fisons MD 800 GC-MS (Manchester, UK) fitted with an on-column injector. The GC-MS was operated in scan mode with an ionizing voltage of 70 eV. The GC was fitted with a capillary column similar to that described above. Helium was used as the carrier gas.

To locate the double-bond positions, dimethyl disulfide (DMDS) adducts of monounsaturated fatty acids (as FAME) were formed for selected samples by treating the total FAME with DMDS (Dunkelblum *et al.*, 1985; Nichols *et al.*, 1986). Adducts were then extracted using hexane/chloroform (4:1 v/v) and analyzed by GC-MS as described elsewhere (Gutierrez *et al.*, 1999).

For spectrofluorometry, fluorescent intensities were measured in a SLM-Aminco Bowman Series 2 luminescence spectrometer (SLM Instruments, IL, USA) using 10 x 10 mm PMMA 4-sided cuvettes with the following instrument conditions: excitation wavelength (Ex) = 340 nm; fluorescence emission wavelength (Em) = 440 and 490 nm. Cells were kept in suspension during measurements using a cuvette stirrer. Cuvette temperature was maintained at 28°C by a circulating water bath (Thermoline, Sydney) to reduce the possibility of fluidity changes occurring as a result of a change in temperature.

The spectrofluorometer photomultiplier gain was optimized using labeled cells with Ex 340 nm and Em 440 nm. Intensity readings were taken every 30 sec over 180 sec, thus each intensity recorded was the mean of 6 readings. To determine background fluorescence, a labeled cell suspension was filtered (0.22 μm) and fluorescence emission measured as described above. The data files of fluorescence intensity (at 440 and 490 nm) for cells and for medium background were imported into a Microsoft Excel spreadsheet for subtraction of background and calculations of generalized polarization (GP). For each GP data point, excitation and emission scans were also assessed to ensure the calculated GP values were not due to anomalous readings. The GP was calculated as previously described (Parasassi *et al.*, 1990). The theoretical maximum and minimum values of GP are 1.0 and -1.0 respectively. GP can be used as a numerical index of membrane fluidity - the higher the value the lower the fluidity (Parasassi *et al.*, 1990).

RESULTS AND DISCUSSION

Isolation of benzene-sensitive mutants

The mutation frequency for generating benzene-sensitive mutants from this *Rhodococcus* sp. was estimated to be 2.7×10^{-3} using the EMS mutagenesis and mutant selection procedure described. A total of five colonies (< 0.5 mm in diameter) were selected as candidates for benzene-sensitivity. These were tested for their sensitivity to different concentrations of benzene. One mutant, labeled strain M2b, was found to have a 60 - 70% sensitivity to benzene as compared to the *w-t* (results not shown). This mutant was selected for further investigation.

Effect of benzene on the fatty acid profiles of strain M2b

Table 1 shows the six fatty acids that dominated the fatty acid profiles of strain M2b when the cells were grown on MM containing mannitol, benzene, and both carbon sources, as obtained from two separate duplicate experiments, Experiment 1 and Experiment 2. The results of Experiment 2 are used simply to show the consistency in the fatty acid trends, henceforth, the discussion will focus on Experiment 1.

The total level of saturated fatty acids changed from 53.3 ± 1.6% for mannitol-grown cells (no benzene) to 60.3 ± 2.1% for benzene-grown cells (no mannitol). The total level of unsaturated fatty acids changed, respectively, from 46.7 ± 1.3% to 39.7 ± 2.7%. All of the fatty acids (16:0, 10Me18:0, and 16:1ω7c) that were found to increase the sat:unsat ratio of the *w-t* from 1.1 (for mannitol-grown cells) to 1.5 (for benzene-grown cells) (Gutierrez *et al.*, 1999), were also shown to have the same effect in strain M2b under identical growth conditions.

Interestingly, strain M2b did not increase its sat:unsat ratio to a higher level during growth in MM containing both carbon sources (Table 1) – a growth condition that has been shown to effectively expose the cells to higher concentrations of benzene (143 to 154 mg/L) (Gutierrez *et al.*, 1999). This ratio was 1.4 with M2b. Under identical growth conditions, it was 1.8 for the *w-t* (Gutierrez *et al.*, 1999). Thus, the mutant did not show the dose-related increase in fatty acid saturation that had previously been observed with the *w-t* (Gutierrez *et al.*, 1999), and which has also been observed in a *Rhodococcus opacus* when exposed to toluene and phenol (Tsitko *et al.*, 1999). The lower sat:unsat ratio achieved by strain M2b appears to be due to a decrease in the abundance of myristic acid (by 39.4%) and increase in oleic acid (by 22.0%) compared to that in the *w-t* when

grown under identical conditions (Gutierrez *et al.*, 1999). Hence, a mutation in the expression of one or both of these fatty acids may account partly or totally for M2b's sensitivity to benzene, and either or both of these fatty acids may contribute a role in providing the *w-t* with some degree of tolerance to benzene. Mannitol, along with benzene, may have contributed to the changes in the sat:unsat ratios observed when both compounds were present in the medium. However, at the concentrations which it was used (1%), this readily metabolizable and relatively innocuous sugar would have an insignificant effect on the fatty acid saturation compared to that of benzene.

Sampling each culture for fatty acid analysis when the optical density reached approximately 0.4 minimized the artifact that fatty acid compositional variation might be due to cells being analyzed at a different stage of growth. Hence, the fatty acid changes observed are concluded to be effectively due to the difference in the treatment (*i.e.* presence or absence of benzene). Since no changes in the ratio of *cis* to *trans* monounsaturated fatty acids were detected, and all unsaturated fatty acids were *cis* monoenoic, the results show that saturated fatty acids were the main components involved in changes observed with the sat:unsat ratio in response to benzene.

Effect of benzene on the membrane fluidity of the w-t

Table 2 shows the effect of benzene on the GP value of the membranes in cells of the *w-t* when grown for 18 to 20 hours on MM containing mannitol, benzene, and both carbon sources, as obtained from duplicate cultures – Experiments 1 and 2. The results obtained from the duplicate, culture 2, are simply shown to demonstrate the consistency in the GP trends. Hence, the discussion will focus on the results from Experiment 1.

The increase in the GP value of the membranes, as induced by the presence of benzene, is correlated with a decrease in membrane fluidity (Parasassi *et al.*, 1990; Learmonth and Gratton 2002). Theoretically, an increase in the sat:unsat ratio of fatty acids has been proposed to lower the fluidity of membranes and reduce the accumulation of solvents at this cellular site (Sikkema *et al.*, 1995; Weber and de Bont, 1996). Therefore, this benzene-induced decrease in membrane fluidity complements our previous results where we showed benzene to induce an increase in the ratio of sat:unsat fatty acids (Gutierrez *et al.*, 1999). These types of fatty acid changes have been associated with decreasing the fluidity of cell membranes in various microorganisms when exposed to toxic organic solvents (Sikkema *et al.*, 1995; Weber and de Bont, 1996), as well as in model phospholipid membrane systems (Cullis and Hope, 1985). No fluorescence was detected in the laurdan-uninoculated controls (data not shown) which eliminates the existence of background fluorescence by other components of cellular or culture medium origin.

Benzene (200 mg/L) was found to have an almost immediate effect on the fluidity of the membranes in the *w-t* strain (Figure 1). After only 0.5 hr incubation, the GP decreased. This correlated with an increase in the fluidity of the membranes. Although there are very few reports available describing the position of benzene in a membrane (Simon *et al.*, 1982; Ward *et al.*, 1986; Boden *et al.*, 1991), its influence at this site is widely accepted to produce an increase in fluidity (Sikkema *et al.*, 1994). A decrease in the fluidity of the cell membrane, to counter the fluidizing effect of the benzene, was not evident within the duration (6 hours) of this experiment. Since fluidity was observed to decrease after the cells had been exposed to the benzene for

approximately 18 to 20 hr (Table 2), this suggests that an adaptive response, set to mitigate benzene toxicity, is induced only after long exposure. With respect to the organism's tolerance during the first 6 hr, this *Rhodococcus* may have a well-adapted inherent mechanism to survive an initial challenge with benzene. Such a mechanism would be beneficial to the organism at times when 'shock' concentrations of benzene, and other toxic solvents, enter the vicinity this organism in the environment.

In this study we have shown that a *Rhodococcus* species produces benzene-induced changes in the sat:unsat ratio of fatty acids, influenced particularly by myristic and oleic acids. These changes may be associated with mitigating solvent toxicity in the cell membrane interface, thus enabling this organism to exist at contaminated sites. The presence of solvent-tolerant microorganisms at contaminated sites is of great ecological benefit to the environment because they can contribute to the natural bioremediation or detoxification of these compounds – a process that involves the microbial conversion of the contaminants into innocuous products.

Acknowledgment

We wish to thank Dr. Peter Fahy and Dorothy Noble for providing us with useful advice on lipid extraction, and Rene Schneider and his laboratory staff for valuable assistance. This work was supported by a Special School of Microbiology and Immunology Research grant at the University of NSW.

References

BODEN, N., JONES, S. A., and SIXL, F. 1991. On the use of deuterium nuclear magnetic resonance as a probe of chain packing in lipid bilayers. *Biochem* 30:2146-2155.

CLAUS, D. and WALKER, N. 1964. The decomposition of toluene by soil bacteria. *J Gen Microbiol* 36:107-122.

CULLIS, P. R. and HOPE, M. J. 1985. Physical properties and functional roles of lipid membranes. In Vance and Vance, ed, *Biochemistry of Lipids and Membranes*. Benjamin/Cummings Publishing Company, Inc, California, pp 25-72.

DUNKELBLUM, E., TAN, S. H., and SILK, R. J. 1985. Double-bond location in monounsaturated fatty acids by dimethyl disulfide derivatization and mass spectrometry. *J Chem Ecol* 11:265-277.

EISENSTADT, E., CARLTON, B. C., and BROWN, B. J. 1994. Gene manipulation. In Gerhardt, ed, *Methods for General and Molecular Bacteriology*. American Society for Microbiology, Washington, DC, pp 297-316.

GUTIÉRREZ J. A., NICHOLS, P., and COUPERWHITE, I. 1999. Changes in whole cell-derived fatty acids induced by benzene and occurrence of the unusual 16:1ω6c in *Rhodococcus* sp. 33. *FEMS Microbiol Lett* 176:213-218.

Appl Environ Microbiol 65:853-855.

HAMILTON, J. T. G., MCROBERTS, W. C., LARKIN, M. J., and HARPER, D. B. 1995. Long-chain haloalkanes are incorporated into fatty acids by *Rhodococcus rhodochrous* NCIMB 13064. *Microbiol* 141:2611-2617.

HEIPIEPER, H. J., WEBER, F. J., SIKKEMA, J., KEWELOH, H., and DE BONT J. A. M. 1994. Mechanisms of resistance of whole cells to toxic organic solvents. *Trends in Biotechnol* 12:409-415.

LEARMONTH, R. P. and GRATTON, E. 2002. Assessment of membrane fluidity in individual yeast cells by laurdan generalized polarization and multi-photon scanning fluorescence microscopy. pp 241-252 *in* R Kraayenhof, AJWG Visser and HC Gerritsen, (Eds.) Fluorescence Spectroscopy, Imaging & Probes: New Tools in Chemical, Physical and Life Sciences, Springer, Heidelberg.

MIDDLEHOVEN, W., KOOREVAAR, M., and SCHUUR, G. 1992. Degradation of benzene compounds by yeasts in acidic soils. *Plant and Soil* 145:37-43.

NICHOLS, P. D., GUCKERT, J. B., and WHITE, D. C. 1986. Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulfide adducts. *J Microbiol Methods* 5:49-55.

PAJE, M. L., NEILAN, B., and COUPERWHITE, I. 1997. A *Rhodococcus* species that thrives on medium saturated with liquid benzene. *Microbiol* 143:2975-2981.

PARASASSI, T., DE STASIO, G., D'UBALDO, A., and GRATTON, E. 1990. Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence. *Biophys J* 57:1179-1186.

SIEGRIST, R. L. 1992. Volatile organic compounds in contaminated soils: the nature and validity of the measurement process. *J Haz Mat* 29:3-15.

SIKKEMA, J., DE BONT, J. A. M., and POOLMAN, B. 1994. Interactions of cyclic hydrocarbons with biological membranes. *J Biol Chem* 269:8022-8028.

SIKKEMA, J., DE BONT, J. A. M., and POOLMAN, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev* 59:201-222.

SIMON, S. A., MCDANIEL, R. V., and MCINTOSH, T. J. 1982. Interaction of benzene with micelles and bilayers. *J Phys Chem* 86:1449-1456.

TSITKO, I. V., ZAITSEV, G. M., LOBANOK, A. G., and SALKINOJA-SALONEN, M. S. 1999. Effect of aromatic compounds on cellular fatty acid composition of *Rhodococcus opacus*.

WARD, A. J. L., RANANAVARE, S. B., and FRIBERG, S. E. 1986. Solvation changes induced in alyotropic liquid crystal containing solubilized benzene. *Langmuir* 2:373-375.

WEBER, F. J., and DE BONT, J. A. M. 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochem Acta* 1286:225-245.

.

Table 1. Compositional (%) changes of six dominant fatty acids in the benzene-sensitive mutant, M2b, when grown on mannitol, benzene, and mannitol+benzene in MM. Data shown are for two separate experiments.

	Experiment 1			Experiment 2		
Fatty acid	Man	Benz	Man+Benz	Man	Benz	Man+Benz
	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD
14:0	15.5 ± 0.4	10.5 ± 0.2	9.4 ± 1.9	13.8 ± 2.5	10.9 ± 0.3	10.5 ± 0.4
16:0	27.1 ± 0.6	39.1 ± 0.9	37.1 ± 1.2	28.7 ± 1.8	42.0 ± 0.4	36.7 ± 0.3
10Me18:0	3.4 ± 0.2	6.2 ± 0.4	7.0 ± 1.4	4.2 ± 1.3	6.2 ± 0.3	6.1 ± 0.1
Sum Sat :	53.3 ± 1.6	60.3 ± 2.1	58.1 ± 6.5	54.6 ± 7.4	64.6 ± 1.6	57.6 ± 1.6
16:1ω7c	12.0 ± 0.3	4.0 ± 0.1	4.5 ± 0.8	10.9 ± 1.7	4.5 ± 0.0	4.8 ± 0.1
16:1ω6c	13.6 ± 0.3	11.1 ± 0.3	12.6 ± 2.2	12.0 ± 1.9	9.0 ± 0.1	13.9 ± 0.1
18:1ω9c	16.4 ± 0.3	18.8 ± 0.3	20.0 ± 1.4	17.8 ± 1.9	19.1 ± 0.1	19.4 ± 0.1
Sum Unsat :	46.7 ± 1.3	39.7 ± 2.7	41.9 ± 5.4	45.4 ± 6.2	39.9 ± 1.1	42.4 ± 0.7
Other:	0.0 ± 0.0	1.9 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sat/Unsat ratio:	1.1	1.5	1.4	1.2	1.6	1.4
Total:	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0

Avg. = average percentage from three replicates.

SD = standard deviation of the replicates.

Man = mannitol-grown cells; Benz = benzene-grown cells; Man+Benz = mannitol+benzene-grown cells.

Table 2. Generalized polarization (GP) of laurdan-labeled cells of the *Rhodococcus w-t* during growth in MM supplemented with mannitol, benzene, and mannitol+benzene.

Treatment	Experiment 1	Experiment 2
Man	0.51 ± 0.002	0.49 ± 0.001
Benz	0.60 ± 0.005	0.58 ± 0.003
Man+Benz	0.64 ± 0.005	0.60 ± 0.002

Note: Experiment 2 is a duplicate of Experiment 1.

Man = mannitol-grown cells; Benz = benzene-grown cells;

Man+Benz = mannitol+benzene-grown cells.

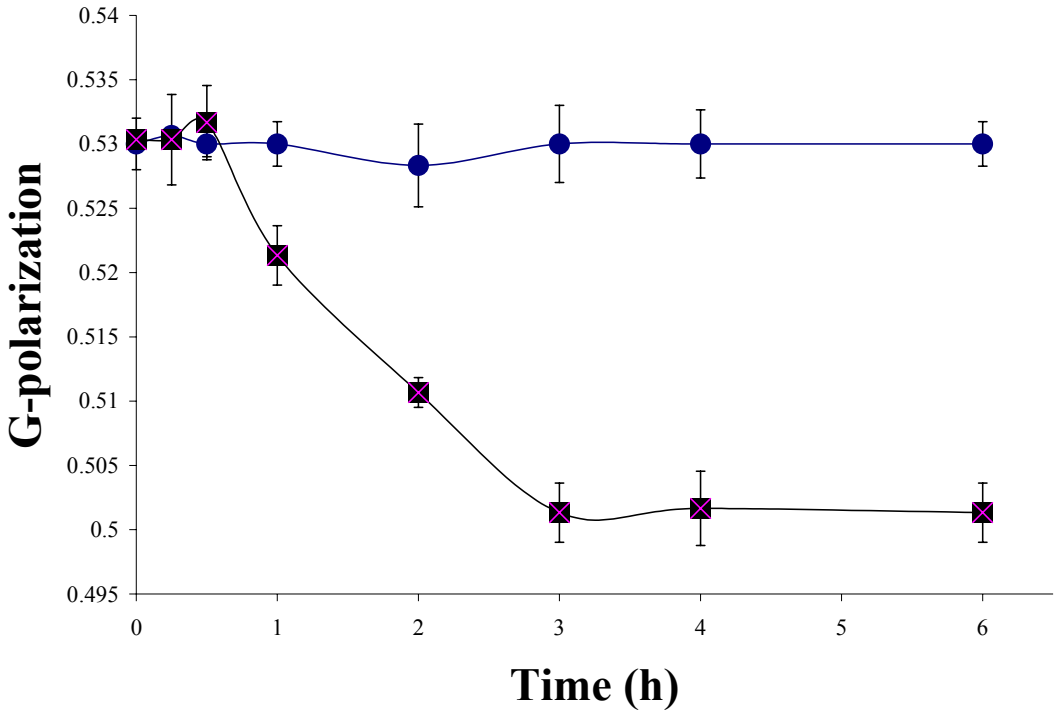

Figure 1. Change in the generalized polarization (GP) value of laurdan-labeled cells of the *Rhodococcus w-t* during exposure to 200 mg/L benzene (■) in MM containing 1% (v/v) mannitol, compared to the benzene-unexposed control (●).