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ORIGINAL PAPER

Membrane fatty acids adaptive profile in the simultaneous presence of arsenic and toluene in *Bacillus* sp. ORAs2 and *Pseudomonas* sp. ORAs5 strains

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Abstract *Bacillus* sp. ORAs2 and *Pseudomonas* sp. ORAs5, two arsenic-resistant bacterial strains previously isolated from sediments of the Orbetello Lagoon, Italy, were tested for their adaptation to mixed contaminants on the level of membrane fatty acid composition. The two bacterial strains were characterized by high levels of arsenic resistance, and *Pseudomonas* sp. ORAs5 was also shown to be solvent-tolerant. The bacterial strains were exposed to mixtures of two toxic compounds: arsenic at fixed concentrations and toluene in variable amounts or, alternatively, toluene at constant values along with arsenic added at variable concentrations. Both strains react to the contaminants by changing the composition of their membrane fatty acids. *Bacillus* sp. strain ORAs2 showed a correlation between growth rate decreases and fatty acids degree of saturation increases in both cases, although pointedly in the presence of 1, 2, and 3 mM of toluene and different additions of arsenic, counteracting membranes fluidity induced by toxic compounds. In *Pseudomonas* sp. ORAs5, adaptive changes in membrane composition was observed both in terms of increases in the degree of saturation and in the *trans*/*cis* ratio of unsaturated fatty acids in the presence of varying toluene and constant arsenic concentrations, whereas only minor changes occurred with increasing arsenic and constant toluene concentrations. Thus, on the level of membrane composition, *Bacillus* sp. ORAs2 showed a higher potential for adaptation to the presence of mixed pollutants, suggesting its probable suitability for bioremediation purposes.

Keywords *Bacillus* · *Pseudomonas* · Fatty acids · Membrane fluidity · Homeoviscous adaptation · *cis*/*trans* isomerization · Orbetello Lagoon

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Introduction

Microorganisms of polluted environments frequently interact with dangerous elements and chemicals with toxic properties, with their cell membranes representing the main target (Heipieper et al. 1994; Weber and de Bont 1996). Environmental stresses show their detrimental effect by increasing the fluidity of the cell membrane of microorganisms (Heipieper et al. 1991; Sikkema et al. 1994). An increase in membrane fluidity may lead to damages, triggering a non-specific permeability, with inhibition of membrane activity (Diefenbach and Keweloh 1994). Proton permeability of the membrane, an important parameter for viability, is also threatened (van de Vossenberg et al. 1999).

Bacterial cells are able to adapt to changes in their membrane fluidity mainly by modifying their membrane fatty acid composition in order to maintain membrane fluidity at a constant level (Petersen and Klug 1994). This mechanism is known as "homeoviscous adaptation", based on changes in the fatty acid composition of membrane lipids (Sinensky 1974; Suutari and Laakso 1974). Regulating membrane fluidity against membrane-active substances mainly consists in changes in the fatty acid

composition of the cell membrane and, in particular, in a modification in the degree of saturation (Diefenbach et al. 1992; Heipieper et al. 1992, 1994; Keweloh et al. 1991). Additionally, some bacteria, mainly *Pseudomonas* and *Vibrio* strains adapt to an increase in their membrane fluidity by an isomerisation of their *cis* to *trans* unsaturated fatty acids (Heipieper et al. 1992, 2003). Because of the steric differences between the two configurations of unsaturated fatty acids, conversion of *cis* into *trans* unsaturated fatty acids reduces membranes fluidity and counteracts against the stress (Seelig and Waespe-Šarcevic 1978; Killian et al. 1992; Heipieper et al. 2003). So far this adaptive mechanism was only observed in strains of the genera *Vibrio* and *Pseudomonas* (Guckert et al. 1986; Heipieper et al. 2003). The *trans* fatty acids are formed by direct isomerization of the complementary *cis* configuration of the double bond without a shift position (von Wallbrunn et al. 2003).

Characteristics of bacterial membrane fatty acids adaptations to environmental stresses suggest a possible use as bioindicator of environment changes (Heipieper et al. 2003; Kaur et al. 2005). This could be applied in detection of toxicity and environmental stress during in situ bioremediation processes, or in determining toxicity in situation in which growth-dependent tests cannot be performed as in natural habitats (Heipieper et al. 1996).

Bacteria involved in bioremediation processes face frequently with more than one contaminant, as in polluted environments very often toxic elements, and organic contaminants are present at the same time. In the United States, 37% of all sites polluted with organic compounds also contain toxic inorganic contaminants, such as heavy metals (Springael et al. 1993).

Several arsenic-resistant bacterial strains were isolated from polluted sediments of the Orbetello Lagoon, Italy, where contaminations by heavy metals, metalloids, and organic solvents were detected. Arsenic contamination in the Orbetello Lagoon which can be up to 100 mg kg^{-1} in the sediments is mostly due to ash, dust and debris originating from a fertilizer production plant located on a bank of this lagoon (Focardi 2005; Pepi et al. 2007). These bacterial strains were isolated and characterized in 2005 with the aim to use them in bioremediation processes of the same native sediments. Among them, *Bacillus* sp. strain ORAs2 and *Pseudomonas* sp. ORAs5 showed higher levels of arsenic resistance both to the trivalent (MICs of 16.68 mM) and pentavalent (MICs > 135 mM) forms (Pepi et al. 2007).

The aim of this work was to investigate the membrane fatty acid adaptability in *Bacillus* sp. ORAs2 and *Pseudomonas* sp. ORAs5, as respect to contemporary additions of the most toxic form of arsenic, the trivalent one, and of toluene. Mechanisms of the membrane fatty acids adaptation, in terms of changes in their degrees of saturation or *cis*/*trans* isomerization of the fatty acids double bonds were tested, as possible bioindicators of environmental stresses during possible bioremediation processes, and in order to test the adaptability of the two bacterial strains to mixed contaminants.

Materials and methods

Microorganisms and culture conditions

Bacillus sp. ORAs2 and *Pseudomonas* sp. ORAs5 were previously isolated as arsenic-resistant microrganisms from polluted sediments of the Orbetello Lagoon, Italy (Pepi et al. 2007). The strains were cultivated in YEPG medium containing (per litre of double distilled water): 5 g of tryptone, 2.5 g of D-glucose, and 2.5 g of yeast extract.

Tests of growth of bacterial strains in the presence of monoaromatic compounds

Different monoaromatic compounds were added at a concentration of 0.1% to the two different bacterial strains. Monoaromatic compounds were the following: benzene, toluene, ethylbenzene, and *o*-xylene. Cultures were arranged in YEPG medium, 10 ml were distributed in 18 ml test tubes sealed with screw-top caps. Inocula (1:100) were carried out with overnight cultures of the two bacterial strains, and cultures were incubated in a rotary drum for 24 h at 28°C. Growth was detected by measuring the turbidity of the cultures.

Growth in the presence of increasing concentrations of toluene

The two bacterial strains were tested for growth in the presence of different concentrations of toluene: 0, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 mM. Cultures were arranged in YEPG medium, 10 ml were distributed in 18 ml test tubes sealed with screw-top caps. Inocula (1:100) were carried out with overnight cultures of the two bacterial strains, and cultures were incubated for 24 h at 28°C, in a rotary drum. Growth was detected by measuring the optical density at 560 nm by an UV-spectrophotometer (Jenway, mod. AC30), and values of percentage of growth were reported, by using growth in the absence of toluene as the 100%.

Growth of the bacterial strains in the presence of mixed toxic compounds, measurement of growth and growth inhibition

Inocula from an overnight culture of each bacterial strain were transferred to three series of 20 ml of YEPG fresh medium. After 3 h of exponential growth, toxic compounds at fixed concentrations: 1, 2, and 3 mM of toluene were added separately to the three series and, at the same time, different concentrations of trivalent arsenic were added to each series. Arsenic concentrations were the followings: 0, 100, 250, 500, 750, 1000, 1250, and 1500 mg l^{-1}. For other three series of 20 ml of YEPG fresh medium treated as above, constant trivalent arsenic concentrations: 200, 400, and 600 mg l^{-1}, were added along with different toluene concentrations: 0, 0.38, 0.75, 1.0, 1.89, 3.0, 3.78, and 5.67 mM, after 3 h of exponential growth. Cultures were grown in a horizontally shaking water bath at 28°C. Cell growth was measured by monitoring the turbidity (optical density at 560 nm) of cell suspensions using a spectrophotometer.

Growth determination in the presence of both toxic compounds

A quantification of the growth inhibition of both bacteria grown in the presence of both toxic compounds in the tested concentrations was carried out. The two bacterial strains were inoculated, with an inoculum 1:100, into flasks containing 20 ml of YEPG medium, and both toxic compounds of the previous experiment, with constant toluene and variable arsenic concentrations, and constant arsenic and variable toluene concentrations, were added at the same time of the inoculum, and incubated at 28°C with shaking. At different time aliquots were harvested and the optical density at 560 nm and the CFU per millilitre of culture were determined. These data were used to evaluate the growth rates μ (h^{-1}) of the two bacterial strains. Growth inhibition caused by toxic compounds was measured by comparing the differences in growth rates μ (h^{-1}), between intoxicated cultures (μ_1) with that of a control culture (μ_0). The growth inhibition of different concentrations of the tested toxic compounds was defined as the percentage of the growth rates of cultures grown with toxic compounds and that of control cultures without addition.

$$\text{Growth rate } (\% \text{ of control}) = \frac{\mu_1}{\mu_0} \times 100$$

All experiments were carried out in triplicate. The average data of these results are shown. The standard deviation was less than 5%.

Lipids extraction and transesterification

The bacterial lipids were extracted from wet pellets of cells of 20 ml suspensions (about 1.5×10^{10} cells). Cells were centrifuged 3 h after addition of the toxic compounds and washed with phosphate buffer (50 mM; pH 7). The lipids were extracted with chloroform–methanol-water as described by Bligh and Dyer (1959). Fatty acid methyl esters were prepared by a 15 min incubation at 95°C in boron trifluoride–methanol (Morrison and Smith 1964). The fatty acid methyl esters were extracted with hexane.

Analysis of fatty acid composition by GC-FID

Analysis of FAME in hexane was performed using a quadruple GC System (HP5890, Hewlett & Packard, Palo Alto, USA) equipped with a split/splitless injector. A CP-Sil 88 capillary column (Chrompack, Middelburg, The Netherlands; length, 50 m; inner diameter, 0.25 mm; 0.25 µm film) was used for the separation of the FAME. GC conditions were: Injector temperature was held at 240°C, detector temperature was held at 270°C. The injection was splitless, carrier gas was He at a flow of 2 ml min^{-1}. The temperature programme was: 40°C, 2 min isothermal; 8°C min^{-1} to 220°C; 15 min isothermal at 220°C. The pressure programme was: 27.7 psi (=186.15 kPa), 2 min isobaric; 0.82 psi min^{-1} (5.65 kPa min^{-1}) to the final pressure 45.7 psi; 15.55 min isobaric at 45.7 psi (310.26 kPa). The peak areas of the carboxylic acids in total ion chromatograms (TIC) were used to determine their relative amounts. The fatty acids were identified by GC and co-injection of authentic reference compounds obtained from Supelco (Bellefonte, USA). The degree of saturation of the membrane fatty acids was defined as the ratio between the three saturated fatty acids (14:0, 16:0, 18:0) and the unsaturated fatty acids (16:1Δ9*cis*, 18:1Δ9*cis*, 18:1Δ11*cis*) present in these bacteria.

Results

Effects of monoaromatic compounds on bacterial growth

Toxicity of benzene, toluene, ethylbenzene and *o*-xylene was tested in the two bacterial strains *Bacillus* sp. ORAs2 and of *Pseudomonas* sp. ORAs5. *Bacillus* sp. strain ORAs2 resulted more sensitive to the different monoaromatic compounds than *Pseudomonas* sp. ORAs5. In the presence of toluene high levels of growth were reached in both bacterial strains (Table 1). *Pseudomonas* sp. strain ORAs5 resulted to be solvent tolerant, as growth at quite high

Table 1 Growth of *Bacillus* sp. ORAs2 and *Pseudomonas* sp. ORAs5 in the presence of 0.1% (v/v) of BTEX monoaromatic compounds

Monoaromatic compound	*Bacillus* sp. ORAs2	*Pseudomonas* sp. ORAs5
Benzene	+	+++
Toluene	++	+++
Ethylbenzene	+	+++
o-Xylene	±	±

Growth was detected as turbidity of cultures

levels was detected in the presence of all the monoaromatic compounds (Table 1).

Effects of toluene and arsenic on bacterial growth

Populations of cells of *Bacillus* sp. ORAs2 and *Pseudomonas* sp. ORAs5 were tested in the presence of different concentrations of toluene and arsenic in order to determine their exact concentration range of toxicity. Arsenic was chosen for such experiments as it is the most abundant and toxic metalloid in the Orbetello Lagoon (Focardi 2005). Toluene was applied as a major toxic component of petroleum hydrocarbons present in the sediments of this area (Focardi 2005). The cells were grown in complex medium and the toxic compounds were added in different concentrations to exponentially growing cells. Relative growth in the presence of the toxic compounds were then calculated using the values obtained for the reduced growth rates of the cells and are shown in Fig. 1. The EC50 values leading to a reduction in growth by 50% could be extrapolated from the plots. EC50 for toluene was 4.8 mM for *Pseudomonas* sp. strain ORAs5 and 4.0 mM for ORAs2 of *Bacillus* sp. (Fig. 1a). EC50 for arsenic was 550 mg l^{-1} for *Pseudomonas* sp. strain ORAs5 and 700 mg l^{-1} for ORAs2 of *Bacillus* sp. (Fig. 1b). Thus, whereas *Pseudomonas* sp. strain ORAs5 showed a higher tolerance towards toluene, it was more sensitive towards arsenic when compared to *Bacillus* sp. ORAs2.

Effects of mixed contaminations with arsenic and toluene on growth and membrane fatty acid composition of *Bacillus* sp. ORAs2

In order to test the effect of mixtures of contaminants on the bacteria analysed, experiments were carried out with different concentration ratios of the two toxic compounds tested. The amounts applied in these experiments were decided as sub lethal concentrations to avoid immediate cell death. Additionally, the effects of such mixed

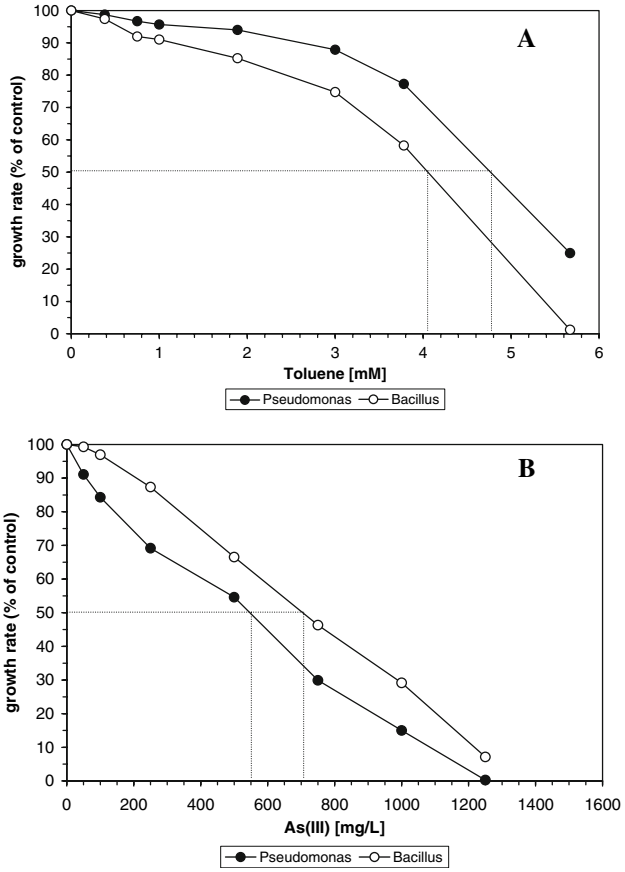

Fig. 1 Effect of toluene (**a**) and arsenic (**b**) on growth of the two bacterial strains *Bacillus* sp. ORAs2 (*open circle*) and *Pseudomonas* sp. ORAs5 (*filled circle*). The *dotted lines* show the EC50 values, the concentrations leading to a 50% growth inhibition, estimated from the curves. Average values, measured standard deviation less than 5%

contaminations on one of the most important adaptive response mechanisms, changes in the degree of saturation of membrane phospholipid fatty acids, were investigated.

Populations of cells of the two series of the bacterial strain *Bacillus* sp. ORAs2 were grown on complex medium containing glucose, and during the exponential growth phase three constant toluene concentrations (1, 2, and 3 mM) were added together with different amounts of arsenic (Fig. 2a); the same was done with three constant arsenic concentrations (200, 400, and 600 mg l^{-1}) together with different amounts of toluene (Fig. 2b).

An additive effect of the two different forms of toxic compounds on the cells can be observed leading to concentration dependent reductions in growth rates at all applied combinations of toluene and arsenic. Similar observations were made for changes in the membrane composition. The degrees of saturation of fatty acids increased with decreasing growth rates, with the highest values for cells that grew with 3 mM of toluene (Fig. 2a) and 600 mg l^{-1} of constant arsenic (Fig. 2a).

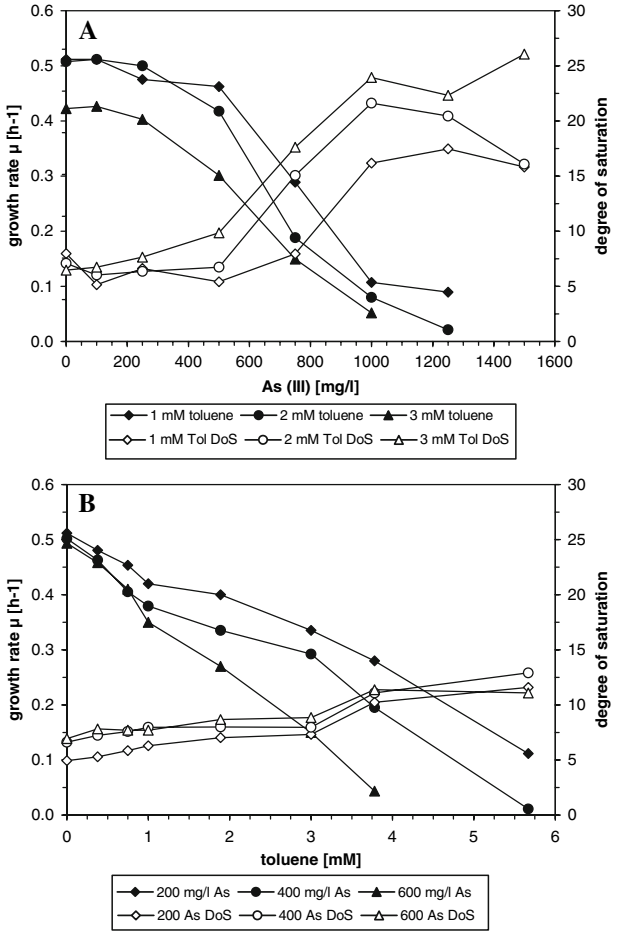

Fig. 2 (*closed symbols*) and degrees of saturation of membrane fatty acids (*open symbols*) of *Bacillus* sp. ORAs2 in the presence of: **a** constant toluene concentrations at 1 mM (*filled diamond*, *open diamond*), 2 mM (*filled circle*, *open circle*) and 3 mM (*filled triangle*, *open triangle*), and of increasing trivalent arsenic concentrations, from 0 to 1,500 mg l^{-1}; and **b** constant trivalent arsenic concentrations at 200 mg l^{-1} (*filled diamond*, *open diamond*), 400 mg l^{-1} (*filled circle*, *open circle*) and 600 mg l^{-1} (*filled triangle*, *open triangle*), and of increasing toluene concentrations, from 0 to 5.67 mM. Average values, measured standard deviation less than 5%

Effects of mixed contaminations with arsenic and toluene on growth and membrane fatty acid composition of *Pseudomonas* sp. ORAs5

The same experiments as previously shown for the *Bacillus* strain were carried out with *Pseudomonas* sp. strain ORAs5. As several strains of the genera *Pseudomonas* contain a special adaptive mechanism on the level of its membrane composition, the *cis–trans* isomerase of unsaturated fatty acids (Heipieper et al. 2003), the *trans/cis* ratios of unsaturated fatty acids are shown instead of the degree of saturation of membrane fatty acids.

Also with *Pseudomonas* sp. strain ORAs5 an additive effect of the two toxic compounds could be shown. However, with this bacterium, slight differences in the cellular response to the toxic compounds could be observed. Only the addition of toluene had a strong increasing effect on the *trans/cis* ratio of unsaturated fatty acids (Fig. 3b), whereas the addition of arsenic only caused a minor adaptive response on the membrane level. Although growth rates depletions were detected for all the three different toluene concentrations added, 1, 2, and 3 mM, the increase of the *trans/cis* ratios were not very big in the presence of all tested arsenic concentrations (Fig. 3a). Similar results were also observed with the degrees of saturation (data not shown).

Discussion

In the present report we presented for the first time a systematic investigation of mixed pollutants, i.e. arsenic and toluene to bacterial strains previously isolated from polluted sediments of the Orbetello Lagoon, Italy, with high arsenic pollution along with the presence of widespread organic contaminants (Donati et al. 2005; Focardi 2005; Pepi et al. 2007). Both bacteria had been shown to be arsenic-resistant (Pepi et al. 2007); here, *Pseudomonas* sp. ORAs5 was also shown to be tolerant towards aromatic organic solvents. However, both bacteria were inhibited in their growth by higher concentrations of the toxic compounds as well as by mixtures of both.

Concerning *Bacillus* sp. strain ORAs2, both situations with mixed arsenic and toluene induced adaptive responses of the cell membranes. Conditions where arsenic was added at increasing concentrations, with constant toluene, have an effect on the membrane adaptive response, as revealed by high levels of the degree of saturation reached. This profile is much evident than that pointed out in the presence of variable toluene and fixed arsenic, albeit with similar trends, as pointed out by lower values of the degree of saturation. The toxicity of toluene is based on the increase of the fluidity of the membranes (Sikkema et al. 1995). Heavy metals action on membranes seems not directly to be involved in the fluidity of phospholipids (Hauser 1991). These elements seem to interact with membrane proteins (Duxbury 1985; Frostegård et al. 1993). Concerning arsenic, the trivalent form is transported into cells by aqua-glyceroporins, the glycerol transport proteins (Stahlberg et al. 2000). In *Bacillus* sp. strain ORAs2 the increase of the degree of saturation is evident at high arsenic concentrations and maybe induced to counteract the increase of arsenic uptake. Concerning other Gram-positive bacteria, in *Rhodococcus erythropolis* DCL14, addition of alkanes, alkanols and terpenes caused a dose-dependent increase in the degree of saturation of the membrane fatty acids as well (de Carvalho et al. 2005).

On strain *Pseudomonas* sp. ORAs5 arsenic has a much lower effect on the membrane adaptive response than

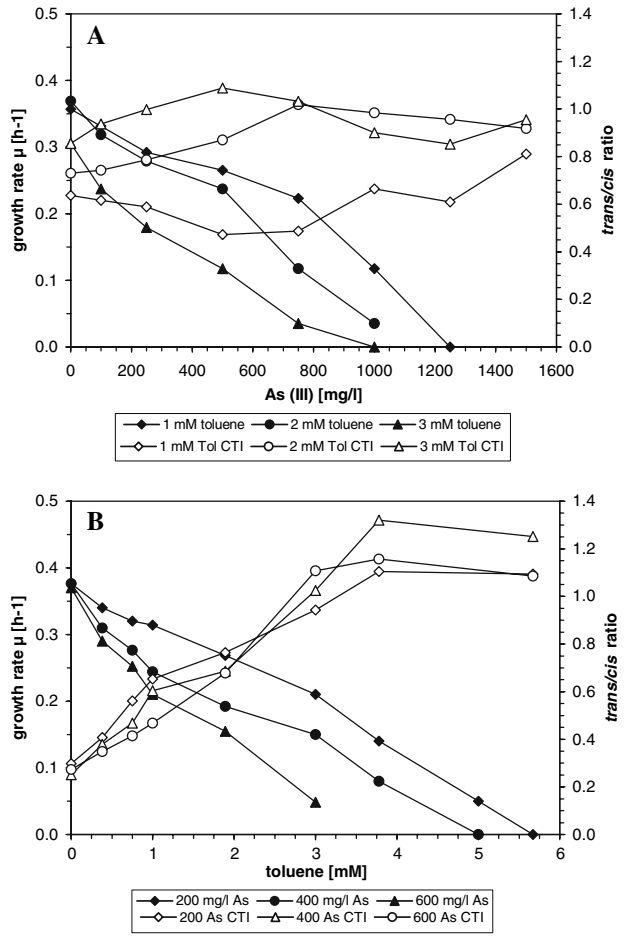

Fig. 3 Growth rates (*closed symbols*) and *trans/cis* ratio (CTI) of unsaturated fatty acids (*open symbols*) of *Pseudomonas* sp. ORAs5 in the presence of: **a** constant toluene concentrations at 1 mM (*filled diamond*, *open diamond*), 2 mM (*filled circle*, *open circle*) and 3 mM (*filled triangle*, *open triangle*), and of increasing trivalent arsenic concentrations, from 0 to 1,500 mg l^{-1}; and **b** constant trivalent arsenic concentrations at 200 mg l^{-1} (*filled diamond*, *open diamond*), 400 mg l^{-1} (*filled circle*, *open circle*) and 600 mg l^{-1} (*filled triangle*, *open triangle*), and of increasing toluene concentrations, from 0 to 5.67 mM. Average values, measured standard deviation less than 5%

toluene. The cells react much stronger to increasing concentrations of toluene than to that of arsenic, as pointed out by *cis/trans* isomerization. It is known that strains of *Pseudomonas putida* reacted to toxic concentrations of toluene by accumulating *trans* unsaturated fatty acids in the membrane instead of the *cis* isomers (Weber et al. 1994). The isomerization of *cis* to *trans* unsaturated fatty acids comprehends strains of the genera *Pseudomonas* including the major representatives *P. putida* and *P. aeruginosa* (Keweloh and Heipieper 1996; von Wallbrunn et al. 2003), and *Vibrio* (von Wallbrunn et al. 2003). Organic contaminants and heavy metals were able to increase *trans/cis* ratio of unsaturated fatty acids in *Pseudomonas putida* S12 (Heipieper and de Bont 1994; Heipieper et al. 1996).

Guckert et al. (1986) have suggested to use a *trans/cis* ratio of unsaturated fatty acids in environmental samples as an index for starvation or pollution stress. This feature could be an interesting way in studying toxicity in natural samples, and to the measurement of toxicity and environmental stress during in situ bioremediation processes. In fact, during bioremediation of polluted sites, the level of *trans* unsaturated fatty acids can be used as a marker for general stress and stress reduction to monitor the biodegradation process (Guckert et al. 1986; Frostegård et al. 1996; MacNaughton et al. 1999). Use of *trans/cis* ratio as biomarker represents a solution to the problem of growth dependency as it also works in non-growing cells, contrary to changes in degree of saturation (Heipieper and de Bont 1994).

According to results of growth of the two bacterial strains in the simultaneous presence of arsenic and toluene, it is highlighted that in a mixed pollution situation *Bacillus* sp. strain ORAs2 seems to be a suitable bacterial strain, at least when looking at the membrane composition and its relative adaptive mechanism. On the other hand *Pseudomonas* sp. strain ORAs5 could give suitable information by using membrane fatty acids as microbial biomarkers in native sediments.

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