

# **Cis/trans isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene**

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**Defence mechanisms of three *Pseudomonas putida* strains growing in the presence of toluene up to 50% (v/v) were investigated. The three strains reacted to toxic concentrations of toluene by accumulating *trans* unsaturated fatty acids in the membrane instead of the *cis* isomers. The membranes of the toluene-adapted cells possessed a higher *trans/cis* ratio and had a higher lipid-ordering since the transition temperature was about 7 centigrade degrees higher compared to the non-adapted cells.**

**Keywords:** *Pseudomonas putida*, *trans* fatty acids, two-phase system, toluene toxicity, membrane fluidity

## **INTRODUCTION**

Aromatic solvents like toluene, styrene and tetralin are toxic for micro-organisms even at subsaturating concentrations in water (de Smet *et al.*, 1978; Sikkema *et al.*, 1992). These solvents partition preferentially in the cell membrane and this excessive accumulation causes expansion of the membrane and impairment of membrane functions (Sikkema *et al.*, 1994). Notwithstanding the clear toxicity of various aromatic solvents to most micro-organisms, bacteria do exist that are able to tolerate high concentrations of such compounds in their environment. Clear examples are *Pseudomonas putida* IH-2000 (Inoue & Horikoshi, 1989) and *P. putida* PpG1 (Shima *et al.*, 1991) which grew in the presence of 50% (v/v) toluene. These strains, however, were not able to metabolize toluene. Recently, two other strains have been isolated which can utilize toxic solvents as carbon and energy source. *P. putida* Idaho grew on toluene and *p*-xylene in concentrations of more than 50% (Cruden *et al.*, 1992) and *P. putida* S12 grew on supersaturating concentrations of styrene (Weber *et al.*, 1993).

The mechanism of this tolerance of these *P. putida* strains is presently unknown, but it is reasonable to expect adaptations at the level of the membrane composition. Changes in the fatty acid composition of membrane lipids may alter the partitioning of solvents in the membrane. An increase of the acyl chain length of the phospholipids from tetradecanoic to octadecanoic acid reduced the partitioning of lindane into liposomes 50-fold (Antunes-Madeira & Madeira, 1989). Incubations of *Escherichia coli* with apolar solvents such as benzene and octanol resulted in increased synthesis of saturated fatty acids (Ingram,

1976; Ingram, 1977). Another adaptation mechanism has been observed in a *Pseudomonas putida* species growing on phenol at high concentrations (Heipieper *et al.*, 1992). These cells adapted by converting the *cis* unsaturated fatty acids into the *trans* isomers which have a higher transition temperature and a decreased fluidity of the membrane.

In this report we describe changes in the fatty acid composition of the phospholipids of the three solvent-tolerant strains *P. putida* PpG1, *P. putida* Idaho and *P. putida* S12, in response to the presence of supersaturating concentrations of organic solvents in the growth medium.

## **METHODS**

**Organism and growth.** *Pseudomonas putida* S12 had been isolated on styrene (Hartmans *et al.*, 1990). *P. putida* Idaho (NRRL B-18435) and *P. putida* PpG1 (ATCC 17453) were obtained from culture collections. Cells were grown in phosphate-buffered (pH 7.0) mineral salts medium (Hartmans *et al.*, 1989) with sodium acetate (60 mM) or glucose (15 mM) as carbon and energy source. Cultures were incubated at 30 °C with horizontal shaking in a water bath (160 oscillations min<sup>-1</sup>, amplitude 2.2 cm).

**Growth in the presence of solvents.** An inoculum of 5% (v/v) from an overnight culture was transferred to fresh medium. When the culture reached the exponential growth phase (OD<sub>660</sub> ≈ 0.4) organic solvents were added. The culture bottles (250 ml) containing 25 ml of medium were closed with Mininert valves (Phase Separations, Waddinxveen, The Netherlands) to prevent evaporation. A partition coefficient of toluene between medium and air of 3.8 (Amoore & Hautala, 1983) was used to calculate the toluene concentrations in the water phase.

**Chemostat.** Cells were grown on mineral salts medium with

**Table 1.** Fatty acid composition (%) of three toluene-tolerant *P. putida* strains grown on glucose, acetate or acetate in the presence of toluene (1%, v/v), respectively

	<i>P. putida</i> S12			<i>P. putida</i> PpG1			<i>P. putida</i> Idaho		
	Glucose	Acetate	Acetate + 1% toluene	Glucose	Acetate	Acetate + 1% toluene	Glucose	Acetate	Acetate + 1% toluene
<b>Fatty acid</b>									
16:0	35.9	31.4	31.2	30.7	34.3	34.5	39.4	38.7	39.5
16:1 <i>trans</i>	4.7	9.6	20.6	12.5	12.3	16.9	4.4	9.0	12.1
16:1 <i>cis</i>	32.8	25.9	13.5	23.2	20.4	9.4	18.9	16.7	2.9
17 cyclo	< 1.0	6.1	5.3	1.3	3.0	7.8	3.0	3.6	6.5
18:0	4.2	1.4	1.3	1.4	3.6	1.4	2.3	2.9	1.7
18:1 <i>trans</i>	1.0	3.1	10.4	1.8	1.8	7.1	4.6	8.7	17.2
18:1 <i>cis</i>	21.4	21.3	16.9	26.7	22.4	19.4	22.0	23.9	8.5
19 cyclo	< 1.0	1.2	< 1.0	1.1	2.1	3.5	5.3	4.3	11.6
<b>Ratio</b>									
Sat./unsaturated	0.67	0.67	0.62	0.54	0.76	0.89	1.00	0.94	1.45
<i>trans/cis</i> 16:1	0.14	0.37	1.5	0.54	0.60	1.8	0.23	0.54	4.1
<i>trans/cis</i> 18:1	0.05	0.15	0.62	0.07	0.08	0.37	0.21	0.36	2.0

15 mM glucose in a fermenter with 700 ml working volume at 30 °C, 350 r.p.m. and at a dilution rate of 0.2 h<sup>-1</sup>. Various toluene concentrations were supplied to the fermenter via the gas phase by passing an adjustable part of the air flow through a column (15 cm) of toluene which was kept at 30 °C. The total air flow was kept constant at 140 ml min<sup>-1</sup>.

**Bacterial survival.** The numbers of viable cells before and after solvents were added to exponentially growing cultures (OD<sub>660</sub> ≈ 0.4) or to cells harvested from steady state chemostat cultures were determined by plating 0.1 ml of suitable dilutions in 0.8% (w/v) saline on solidified agar medium with the same growth substrate (e.g. acetate or glucose) as the liquid cultivation medium.

**Fatty acid analysis.** The total lipids of cells were extracted with chloroform/methanol (Bligh & Dyer, 1959). The fatty acids were methylated using boron trifluoride/methanol according to the method of Morrison & Smith (1964). The fatty acid methyl esters were extracted into hexane and normally analysed on a Chrompack CP9000 gas chromatograph equipped with a CP-Sil 88 WCOT fused-silica column. Fatty acid identities were confirmed by GC-MS analysis using a 5890A Hewlett/Packard gas chromatograph equipped with a CP-Sil 19CB WCOT fused-silica capillary column; mass spectra were recorded on a 5970 series mass-selective detector with a 59822B Ionization Gauge Controller (Hewlett/Packard).

**Phase transition temperature.** The temperature-dependent vibrational frequency of the CH<sub>2</sub>-stretch in lipids of whole bacterial cells was measured by Fourier transform infrared spectroscopy (FTIR). FTIR measurements were carried out using a Perkin-Elmer series 1750 FTIR spectrometer equipped with a 7500 data station, as described by Crowe *et al.* (1989). Exponentially growing cells were harvested and washed twice with water. The pellet was placed between two CaF<sub>2</sub> windows. The sample was cooled to -10 °C and slowly heated; spectra were recorded after every stepwise increase (2 to 3 centigrade degrees) in temperature.

## RESULTS

### Fatty acid composition

The three solvent-resistant strains, *P. putida* PpG1, *P. putida* Idaho and *P. putida* S12 were grown in mineral medium containing either glucose, acetate, or acetate in the presence of toluene (1%, v/v). Exponentially growing cells from these cultures were harvested, washed and the fatty acid profiles were determined (Table 1).

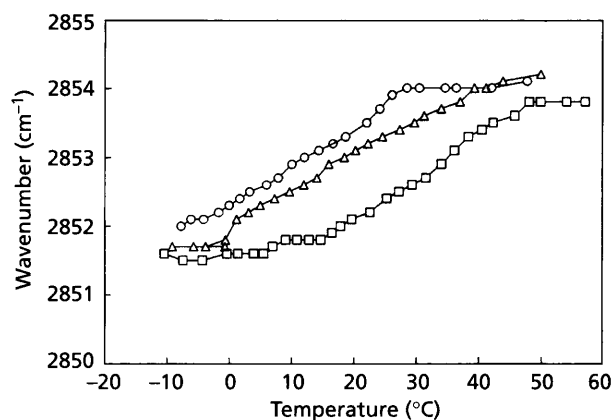
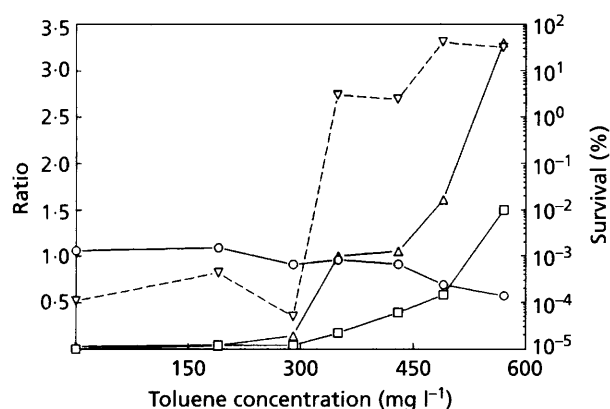
When glucose-grown *P. putida* S12 cells were compared to acetate-grown cells, an increase in the amount of *trans* and a decrease in the *cis* unsaturated fatty acids was seen. A further increase in the *trans/cis* ratio was observed when *P. putida* S12 cells were grown on acetate in the presence of toluene (1%). The acetate-grown cells also had a higher percentage of cyclopropane fatty acids when compared to glucose-grown cells. However, the presence of toluene had no effect on these cyclopropane fatty acids. Similarly, in two other solvent-tolerant strains, *P. putida* PpG1 and *P. putida* Idaho, high *trans/cis* ratios were observed when grown in the presence of high toluene concentrations.

### Stability of the adaptation

Subsequently, we determined whether the observed changes in fatty acid profiles, as dependent on growth conditions, were reversible. *P. putida* S12 grown on acetate in the presence of 1% toluene was used to inoculate (1%) acetate and glucose medium without solvents. The fatty acid composition of the cells grown on acetate was almost identical to the fatty acid composition of the parent culture grown in the presence of toluene. However, a decrease in the *trans/cis* ratio was observed, when grown on glucose (Table 2).

**Table 2.** Fatty acid composition (%) of toluene-adapted *P. putida* S12 grown in the absence of toluene for about 100 generations on glucose or acetate, respectively

	Glucose	Acetate
<b>Fatty acid</b>		
16:0	38.4	32.5
16:1 <i>trans</i>	11.5	25.2
16:1 <i>cis</i>	19.1	12.2
17 cyclo	3.1	2.8
18:0	2.8	2.1
18:1 <i>trans</i>	2.9	6.9
18:1 <i>cis</i>	22.3	16.9
19 cyclo	< 1.0	1.0
<b>Ratio</b>		
Sat./unsaturated	0.79	0.63
<i>trans/cis</i> 16:1	0.60	2.1
<i>trans/cis</i> 18:1	0.13	0.41

**Fig. 2.** Phase transition melting temperature of *P. putida* S12 grown on glucose (○), acetate (△) and acetate medium containing 400 mg toluene l<sup>-1</sup> (□), respectively. Wavenumbers of the CH<sub>2</sub> symmetric stretch in the lipids were measured using FTIR spectroscopy at various temperatures.**Fig. 1.** Effect of toluene on the fatty acid composition of *P. putida* S12 and on survival of cells given a shock addition of toluene. Cells were grown in a glucose-limited chemostat in the presence of various toluene concentrations. Ratio (—) of saturated/unsaturated fatty acids (○), *trans/cis* ratio of hexadecenoic acid (△) and octadecenoic acid (□) and the percentage survivors (---) 1 h after the extra addition of toluene (1%, v/v) (▽).

### Subsaturating concentrations of toluene

Toluene concentrations below the maximum solubility in water (640 mg l<sup>-1</sup> at 30 °C) (Bohon & Claussen, 1951) also slowed the growth of *P. putida* S12 on glucose or acetate medium. A toluene concentration of about 350 mg l<sup>-1</sup> reduced the growth rate to one-half of the maximum. When these cells were transferred to fresh medium with the same toluene concentration, no growth inhibition was observed.

The effect of toluene on the fatty acid composition of *P. putida* S12 was also determined by growing the organism

in a glucose-limited chemostat at a dilution rate of 0.2 h<sup>-1</sup>. Various subsaturating toluene concentrations were applied to the fermenter via the gas phase. The fatty acid composition of the cultures was determined after a steady-state was reached in the chemostat. Toluene concentrations below 300 mg l<sup>-1</sup> in the water phase did not result in changes in the fatty acid composition. However, substantial changes in the *trans/cis* ratio of the unsaturated fatty acids were observed in cells grown in the presence of higher toluene concentrations (Fig. 1). The steady-state chemostat cultures were also used to determine the effect on cell viability of a 'shock' addition of toluene (1%). Only a small number of survivors were recorded in cultures grown in the presence of less than 300 mg toluene l<sup>-1</sup>. However, when grown in the presence of more than 300 mg toluene l<sup>-1</sup>, a high percentage of cells survived this addition of toluene.

### Phase transition temperature

Fatty acids undergoing melting from gel to liquid phase show an adsorption shift of 2–4 wavenumbers in the infrared spectrum (Cameron & Dluhy, 1987). The symmetric vibration band of CH<sub>2</sub> groups at a frequency around 2850 cm<sup>-1</sup> was measured using FTIR spectroscopy. This adsorption maximum was determined at different temperatures for *P. putida* S12 grown on glucose, acetate or acetate medium containing 400 mg toluene l<sup>-1</sup>, respectively. In Fig. 2 it can be seen that cells grown in the presence of toluene possess a lipid melting temperature which is about 7 centigrade degrees higher than in cells grown in the absence of toluene.

### DISCUSSION

Previously we have isolated 14 bacteria on subsaturating concentrations of styrene as sole carbon and energy source (Hartmans *et al.*, 1990). These bacteria were

thought not to grow on supersaturating concentrations of styrene, as these high styrene concentrations were expected to be toxic (de Smet *et al.*, 1978; Sikkema *et al.*, 1994). However, one of the selected strains, *P. putida* S12, eventually grew on styrene at concentrations of more than 50% after a long lag-phase (Weber *et al.*, 1993).

In the present investigation, significant changes in the fatty acid profiles of solvent-adapted strains have been observed. *P. putida* S12 and two other solvent-tolerant strains responded to the presence of 1% toluene by increasing the amount of *trans* fatty acids and by decreasing the corresponding *cis* isomer. This adaptation in *P. putida* S12, occurred not only at supersaturating concentrations but also at subsaturating concentrations of toluene in water. No effects were seen below 300 mg toluene l<sup>-1</sup>, but above 300 mg l<sup>-1</sup> the *trans/cis* isomer ratio of the cells increased dramatically. Cells having a high *trans/cis* isomer ratio were well equipped to survive 'shock' additions of toluene (1%).

The *cis/trans* isomerization as an adaptation mechanism in *P. putida* S12 is quite remarkable as most bacteria adapt to membrane-active compounds by changing the saturation index of their lipids (Ingram, 1976, 1977; Ingram & Buttke, 1984; Keweloh *et al.*, 1990). Although 15 years ago *trans* monounsaturated fatty acids were regarded as non-natural fatty acids (Lehninger, 1977), over the last 10 years *trans* fatty acids in bacteria have been reported in, for instance *Arcobacter cryaerophila* (Moss & Daneshvar, 1992), *P. aeruginosa* (de Andres *et al.*, 1991), *P. atlantica* (Guckert *et al.*, 1987), *P. putida* (Heipieper *et al.*, 1992), *Vibrio cholerae* (Guckert *et al.*, 1986) and other *Vibrio* species (Okuyama *et al.*, 1990), a marine isolate (Gillan *et al.*, 1981) and in methane-utilizing bacteria (Makula, 1978). Recently, a *cis/trans* isomerization has been observed as an adaptation mechanism of a *P. putida* strain to toxic phenol concentrations (Heipieper *et al.*, 1992). From our results it now appears that this defence mechanism is widespread in *P. putida* species.

The *trans* isomer has a steric configuration which is similar to that of saturated fatty acids. The *cis*-isomer has a kink in the acyl-chain of the fatty acids, which causes steric hindrance and results in a membrane with a higher fluidity. Studies with *Acholeplasma laidlawii* membranes enriched with exogenously supplied fatty acids have shown that the phase transition temperature between membranes containing about 80% *cis* or *trans* octadecenoic acid, respectively, differ about 45 centigrade degrees (Macdonald *et al.*, 1985). In the toluene-adapted strain possessing a high *trans/cis* ratio, the transition temperature of the membrane *in vivo* was approximately 7 centigrade degrees higher compared to the non-adapted cells. This provides the cell with a mechanism to compensate for loss of membrane integrity as a result of the accumulation of lipophilic compounds. Furthermore, an increased lipid ordering also opposes partitioning of lipophilic compounds in the membrane (Antunes-Madeira & Madeira, 1989).

Apart from an increase in the *trans/cis* ratio of the

unsaturated fatty acids, an increase in the amount of cyclopropane fatty acids was observed in acetate-grown cells when compared to glucose-grown cells. The lipids of acetate-grown *P. putida* S12 consisted of about 6% cyclopropane fatty acids, whereas glucose-grown cells did not contain these fatty acids. However, adaptation to toluene either in batch or in chemostat cultures did not enhance the level of cyclopropane fatty acids.

Growth of *P. putida* S12 in batch-cultures in the presence of supersaturating toluene concentrations was only observed when using toxic concentrations of acetate or propionate as carbon source (Weber *et al.*, 1993). At 60 mM acetate (pH 7.0), about 0.3 mM of the undissociated acetic acid will be present in the medium. Results of Sheu & Freese (1972) suggest that these low concentrations of acetic acid are membrane-active. Acetate-adapted cells had an increased *trans/cis* ratio of the monounsaturated fatty acids. As a result, cells from such cultures had a higher degree of lipid ordering in the membrane and consequently these cells were able to further adapt and grow in the presence of toluene. Nakajima *et al.* (1992) and Aono *et al.* (1992) have shown that toluene-resistant bacteria can be isolated effectively by first adapting bacteria to the presence of a second phase of the less toxic solvent, xylene. Pre-adaptation of cells to less toxic compounds (acetate, xylene) or to subsaturating concentrations of toxic solvents (toluene) seems to be an effective technique to isolate solvent-tolerant microorganisms.

*P. putida* S12 cells possessing a high *trans/cis* isomer ratio after growth on acetate medium containing toluene, did not revert back to normal fatty acid content upon several generations growth without toluene. This response seems to be quite unusual, but these cells were grown on a medium with an acetate concentration (60 mM) which is normally toxic for the cells. Apparently, the modification in the fatty acid composition induced by toluene, is of benefit for the cells to grow in the presence of toxic acetate concentrations. Upon removal of acetate, a normal fatty acid composition was observed, indicating that the *trans/cis* isomerization is an adaptation mechanism and not a mutation.

The present investigation has focussed only on one possible defence mechanism of *P. putida* strains to toxic solvents. Further investigations will be necessary to determine whether *trans/cis* isomerization of the fatty acids is an important factor in gaining resistance or that other, as yet undiscovered adaptations are of primary importance. In this respect it will be worthwhile to consider the known effects of alcohols on microorganisms including changed protein/lipid ratios or changes in the phospholipid classes (Ingram & Buttke, 1984).

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