

## Cell Envelope Changes in Solvent-Tolerant and Solvent-Sensitive *Pseudomonas putida* Strains following Exposure to *o*-Xylene

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**Solvent-tolerant and -sensitive *Pseudomonas putida* strains were studied to determine their cell envelope changes following exposure to *o*-xylene. Both strains produced *trans*-unsaturated fatty acids. The tolerant strain showed an increase in total fatty acids, an increase in saturated fatty acids, and modified lipopolysaccharide. It is suggested that these envelope modifications aid in survival at high concentrations of organic solvents.**

Recently, several *Pseudomonas* strains with increased tolerance to organic solvents have been isolated (5, 10, 12, 23). Researchers have suggested that *trans*-unsaturated fatty acids (10, 28), magnesium ions (13, 23), and an alkylhydroperoxide reductase (8) contribute to solvent tolerance. Genes involved in cyclohexane tolerance have been cloned (1, 20), although the products of these genes have not been characterized or used to confer solvent tolerance on strains other than *Escherichia coli*. The mechanism of solvent tolerance is not understood.

*Pseudomonas putida* Idaho grows in biphasic organic-aqueous media with *p*-xylene, *m*-xylene, or toluene as the sole carbon and energy source (5) and in the presence of saturated solutions of *o*-xylene which it cannot degrade. Because one of the immediate effects of exposure to hydrocarbons is membrane damage (7, 24, 25), it is likely that a tolerance mechanism exists at the level of the cell envelope. To more fully understand the cell envelope dynamics of solvent-tolerant organisms, in a side-by-side comparison, the responses of a solvent-tolerant organism and a closely related solvent-sensitive organism were analyzed following exposure to *o*-xylene. This study examined the similarities and differences in phospholipid content, lipopolysaccharide (LPS) structure, and permeability between *P. putida* Idaho and *P. putida* MW1200 (26), which cannot tolerate *o*-xylene in excess of 75 ppm.

### Bacterial strains, culture media, and growth conditions.

Two bacterial strains were used in this study, *P. putida* Idaho (5) and *P. putida* MW1200 (ATCC 39119). Both strains were routinely grown under continuous culture conditions at 25°C in a minimal salts medium (3) supplemented with 10 mM sodium succinate. For each experiment, cells were removed from the continuous culture system and transferred to 250-ml flasks containing fresh minimal salts-succinate medium. *o*-Xylene was added directly to the culture medium at concentrations of 75 and 200 ppm in those experiments which utilized organic solvents. Neither strain can metabolize *o*-xylene (verified by gas chromatographic analysis following each experiment). Each strain was exposed to *o*-xylene for various periods of up to 6 h, harvested by centrifugation, and then lyophilized.

**Lipid extraction and analysis.** Lyophilized cells were extracted, and the fatty acids were derivatized by the method of Mayberry and Lane (17), which was modified by addition of phosphatidylcholine (dinonadecanoic; Avanti Polar Lipids, Inc.) as an internal standard prior to the extraction. The resultant fatty acid methyl esters were then analyzed by gas chromatography-mass spectrometry (Hewlett-Packard 5890 gas chromatograph equipped with a series 5971 mass selective detector) as described previously (21).

Both strains exhibited profiles typical of fluorescent pseudomonads. The total (nonhydroxy and hydroxy) fatty acid content increased in the Idaho strain but decreased in *P. putida* MW1200 after exposure to *o*-xylene (Table 1). The molar percentages of the hydroxy fatty acids did not appear to shift in either strain following *o*-xylene exposure. An increase in the relative percentage of saturated fatty acids was observed in the xylene-exposed Idaho strain, particularly in hexadecanoic acid (16:0) and octadecanoic acid (18:0).

Both strains made *trans*-unsaturated fatty acids following exposure to *o*-xylene. Each strain showed significant increases in 16:1 $\omega$ 7*trans* (Fig. 1) when exposed to 75 ppm *o*-xylene. The Idaho strain demonstrated less of an increase in both 16:1 $\omega$ 7*trans* and 18:1 $\omega$ 7*trans* at 75 ppm than did the MW1200 strain and showed a greater increase at 200 ppm than at 75 ppm. The *cis-trans* conversion was also observed by Weber et al. (28).

**LPS analysis.** LPS was isolated from both strains by the method of Darveau and Hancock (6). The recovered LPS was then analyzed by using a tricine-sodium dodecyl sulfate-polyacrylamide gel (16). LPS bands were visualized by using the silver staining method of Tsai and Frasch (27). A silver-stained polyacrylamide gel showing LPS of each strain grown in the presence or absence of *o*-xylene is shown in Fig. 2. Solvent-sensitive strain MW1200 showed a ladder-like banding pattern indicative of smooth-type LPS (lanes 1 and 2). No significant change in the LPS banding pattern was seen in cells grown in the presence of 75 ppm xylene (lane 2). When this strain was subjected to 200 ppm *o*-xylene and incubated for 30 min, it was difficult to isolate good quality LPS from these cells (lane 3) because of cell lysis (verified by microscopy; data not shown).

*P. putida* Idaho showed typical rough-type LPS when grown in the presence or absence of *o*-xylene. The arrowheads indicate carbohydrate bands that appeared under each condition. A higher-molecular-weight band that was visible in the LPS of the Idaho strain grown in the absence of *o*-xylene disappeared

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TABLE 1. Molar percentages and concentrations of fatty acids from *P. putida* MW1200 and *P. putida* Idaho grown in the presence or absence of *o*-xylene

Fatty acid type or parameter	Strain Idaho in 0 ppm <i>o</i> -xylene	Strain Idaho in 75 ppm <i>o</i> -xylene	Strain Idaho in 200 ppm <i>o</i> -xylene	Strain MW1200 in 0 ppm <i>o</i> -xylene	Strain MW1200 in 75 ppm <i>o</i> -xylene
<b>Hydroxy</b>					
3OH10	1.80 ± 0.72 <sup>a</sup>	1.95 ± 0.65	1.54 ± 0.71	0.95 ± 0.88	2.96 ± 1.81
2OH12	4.85 ± 1.94	4.96 ± 1.81	5.13 ± 1.02	3.93 ± 1.13	5.01 ± 1.18
3OH12	4.01 ± 2.28	4.33 ± 0.98	3.72 ± 0.78	4.59 ± 0.94	5.14 ± 0.78
<b>Saturated</b>					
12:0	0.33 ± 0.04	0.27 ± 0.06	0.52 ± 0.47	0.35 ± 0.19	1.00 ± 0.90
14:0	0.23 ± 0.11	0.27 ± 0.10	0.35 ± 0.11	0.23 ± 0.11	0.26 ± 0.13
15:0	0.13 ± 0.13	0.13 ± 0.10	0.66 ± 0.08	0.08 ± 0.06	0.04 ± 0.09
16:0	28.90 ± 1.77	28.87 ± 1.68	30.54 ± 9.23	32.12 ± 5.80	29.74 ± 3.48
18:0	1.06 ± 0.30	1.51 ± 0.07	2.50 ± 0.33	1.26 ± 0.63	1.64 ± 0.77
<b>Unsaturated</b>					
16:1 $\omega$ 7 $cis$	27.89 ± 0.23	25.54 ± 0.88	16.77 ± 1.5	31.37 ± 0.58	27.01 ± 2.09
16:1 $\omega$ 7 $trans$	1.14 ± 0.20	2.46 ± 0.58	13.29 ± 1.83	2.12 ± 0.72	5.57 ± 0.88
18:1 $\omega$ 7 $cis$	26.82 ± 2.12	26.86 ± 1.74	22.58 ± 2.06	22.64 ± 0.78	21.63 ± 0.92
18:1 $\omega$ 7 $trans$	0.47 ± 0.14	0.32 ± 0.13	4.57 ± 1.67	1.11 ± 0.50	1.68 ± 0.61
Cyclopropyl 17	0.71 ± 0.12	0.60 ± 0.03	0.89 ± 0.24	1.62 ± 1.03	1.31 ± 0.80
Cyclopropyl 19	0.06 ± 0.02	0.25 ± 0.14	0.36 ± 0.19	0.10 ± 0.07	0.74 ± 0.35
Avg saturated-unsaturated fatty acid ratio	0.54	0.55	0.64	0.60	0.60
Avg $trans$ - $cis$ fatty acid ratio	0.03	0.05	0.40	0.07	0.18
Hydroxy fatty acid concn <sup>b</sup>	8.94 ± 0.97	ND <sup>c</sup>	17.1 ± 2.06	11.64 ± 1.80	3.28 ± 0.65
Nonhydroxy fatty acid concn <sup>b</sup>	63.8 ± 6.80	ND	122.4 ± 14.50	81.6 ± 13.6	23.6 ± 4.61

<sup>a</sup> Values are average moles percent ± 1 standard deviation ( $n = 5$ ).

<sup>b</sup> Micromoles per milligram of cell dry weight.

<sup>c</sup> ND, not determined.

from the LPS of the *o*-xylene-exposed cells as the *o*-xylene concentration was increased (lanes 4 and 5, single arrowhead) and was replaced by a lower-molecular-weight band (lane 6, double arrowhead).

**Permeability assay.** Because of the differences in LPS and fatty acid structure between these strains, it was hypothesized that the Idaho strain may be less permeable to *o*-xylene and therefore less susceptible to the effects of toxic hydrophobic compounds. Direct assays of permeability were tried with radiolabeled *o*-xylene, but the results obtained with such assays

were not reproducible. Difloxacin (kindly donated by Abbott Laboratories) is a hydrophobic antibiotic which has an octanol-water partition coefficient of 3.1 (15), equal to that of *o*-xylene. To test each strain's relative permeability, both strains were grown in the presence or absence of *o*-xylene and then exposed to various concentrations of the antibiotic to see whether membrane alterations affect the MIC.

Each strain was grown in the absence of *o*-xylene, pelleted by centrifugation (10 min, 8,000 ×  $g$ ), and then transferred to minimal salts-succinate medium containing various concentrations of difloxacin. After establishment of a MIC for each strain, the experiment was repeated in the same manner but with addition of *o*-xylene at the concentrations used in the previous experiments. Following 3 h of exposure to the solvent, the cells were collected as described above and then the MIC was determined again. The Idaho strain was more resistant to the effects of difloxacin than was strain MW1200, but the membrane adaptations of this strain did not affect the MIC of difloxacin, which was 10  $\mu$ g/ml. The MIC for strain MW1200 was 0.1  $\mu$ g/ml when it was grown in minimal salts-succinate medium. This value dropped to >0.05  $\mu$ g/ml following exposure to *o*-xylene.

**Analysis of cell envelope alteration.** The conversion of unsaturated fatty acids from  $cis$  to  $trans$  has been linked to prevention of membrane damage by decreasing membrane fluidity (9–11, 19, 28). The results of this study show that both solvent-tolerant and -sensitive strains make  $trans$ -unsaturated fatty acids following exposure to organic solvents. Therefore,  $trans$ -unsaturated fatty acids are probably synthesized as a general response to environmental stress and not as a mechanism of resistance.

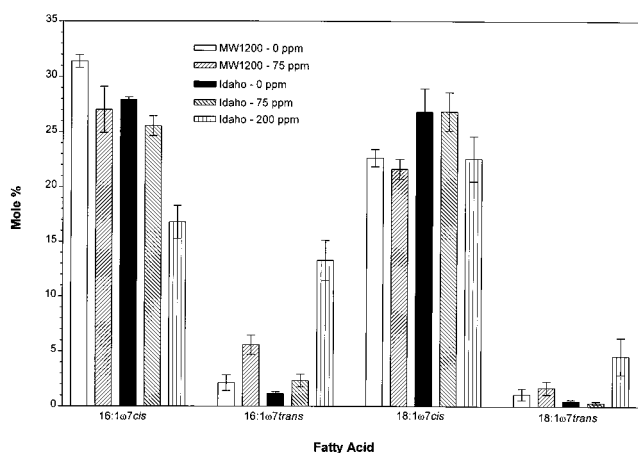


FIG. 1. Mole percentages of  $cis$ - and  $trans$ -unsaturated fatty acids of *P. putida* MW1200 and *P. putida* Idaho when grown in succinate and following exposure to 75 and 200 ppm *o*-xylene. Error bars = 1 standard deviation ( $n = 5$ ).

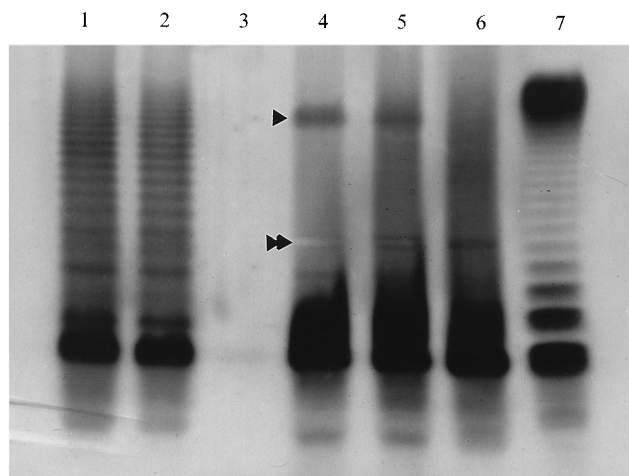


FIG. 2. Silver-stained 14% polyacrylamide gel showing LPS from *P. putida* MW1200 and *P. putida* Idaho. A sample of approximately 1  $\mu$ g of LPS was loaded per lane. Lanes: 1, LPS from *P. putida* MW1200 grown in the absence of *o*-xylene; 2, LPS from strain MW1200 grown in the presence of 75 ppm *o*-xylene; 3, LPS from strain MW1200 exposed to 200 ppm *o*-xylene; 4, LPS of *P. putida* Idaho grown in the absence of *o*-xylene; 5, LPS from the Idaho strain grown in the presence of 75 ppm *o*-xylene; 6, LPS from the Idaho strain grown in the presence of 200 ppm *o*-xylene; 7, *E. coli* LPS (smooth type, for comparison). Electrophoresis was performed with a constant voltage of 65 V for 15 min and then 80 V for 100 min.

The increased total nonhydroxy fatty acid and hydroxy fatty acid content in *P. putida* Idaho following exposure to xylene could indicate that a membrane repair mechanism exists in this strain that allows rapid repair of damaged membrane components. Preliminary evidence that shows a more rapid turnover of phosphatidylglycerol and phosphatidylethanolamine in the Idaho strain than in strain MW1200 has been collected (29). Rapid membrane repair could explain the magnesium ion requirement observed to occur in solvent-tolerant strains (13, 23), as magnesium is known to be one of the important cations in LPS stabilization (14).

While the observed alteration of LPS structure following exposure to xylene did not make *P. putida* Idaho less permeable to a hydrophobic antibiotic, it was much less susceptible than a similar strain which exhibited smooth-type LPS. It is possible that the Idaho strain LPS excludes hydrophobic substrates. Several *Pseudomonas* strains are antibiotic resistant because of changes in the LPS and protein structure of the cell surface which change the cell surface hydrophobicity and permeability properties (2, 4, 18, 22, 24).

Although the full mechanism of solvent tolerance remains unclear, several facts are known about the phenomenon. The results of this study and others (5, 13) show that solvent tolerance occurs in the absence of metabolism of the organic solvent, eliminating rapid substrate metabolism and substrate detoxification as solvent tolerance mechanisms. Although *trans*-unsaturated fatty acids were shown to increase the phase transition temperature in liposomes (25), this study shows that their presence in a cell membrane does not confer solvent tolerance. *P. putida* Idaho is less permeable to hydrophobic substrates than is strain MW1200, likely because of its LPS structure. The increased fatty acid amount per milligram of cell dry weight suggests that a rapid repair mechanism exists in the Idaho strain. There is no genetic information about solvent tolerance in *Pseudomonas* species. Investigators reporting solvent-tolerant strains have failed to isolate plasmid material from these strains (5, 13, 23), indicating that the genetic ele-

ments responsible for solvent tolerance are probably not located on a plasmid. While there is still much to be learned about the phenomenon of solvent tolerance, the information obtained with this study provides useful leads for further experimentation.

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