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Phospholipid Biosynthesis and Solvent Tolerance in *Pseudomonas putida* Strains

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The role of the cell envelope in the solvent tolerance mechanisms of *Pseudomonas putida* was investigated. The responses of a solvent-tolerant strain, *P. putida* Idaho, and a solvent-sensitive strain, *P. putida* MW1200, were examined in terms of phospholipid content and composition and of phospholipid biosynthetic rate following exposure to a nonmetabolizable solvent, *o*-xylene. Following *o*-xylene exposure, *P. putida* MW1200 exhibited a decrease in total phospholipid content. In contrast, *P. putida* Idaho demonstrated an increase in phospholipid content 1 to 6 h after exposure. Analysis of phospholipid biosynthesis showed *P. putida* Idaho to have a higher basal rate of phospholipid synthesis than MW1200. This rate increased significantly following exposure to xylene. Both strains showed little significant turnover of phospholipid in the absence of xylene. In the presence of xylene, both strains showed increased phospholipid turnover. The rate of turnover was significantly greater in *P. putida* Idaho than in *P. putida* MW1200. These results suggest that *P. putida* Idaho has a greater ability than the solvent-sensitive strain MW1200 to repair damaged membranes through efficient turnover and increased phospholipid biosynthesis.

In the past several years, many organic solvent-tolerant organisms have been discovered (1, 8, 20, 30, 35). The phenomenon of solvent tolerance is an important one, because it allows microorganisms to grow in high concentrations of organic solvents. Organisms capable of surviving these conditions have great application in bioremediation of contaminated sites and "end of the pipe" biotransformation of industrial wastes, and many have potential for use in biphasic bioconversion systems (9). Solvent-tolerant *Pseudomonas* strains capable of producing solvent-stable lipases and proteases have been isolated recently, further expanding the utility of these organisms (30, 31).

The mechanism for solvent tolerance is unknown, but some conclusions can be made from the current literature. The toxic effects of aromatic hydrocarbons are associated with loss of cytoplasmic membrane integrity (10, 37–39, 44). This loss of membrane integrity results in disruption of proton motive force, loss of membrane barrier functions, inhibition of membrane protein function, and subsequent cell lysis and death. Solvent tolerance does not appear to be related to degradation, as most solvent-tolerant organisms tolerate a wide variety of aromatic hydrocarbons, alkanes, and alcohols that they are incapable of degrading or transforming (8, 20, 35, 44). Most of these organisms do not harbor plasmids, indicating that the resistance factors reside on the chromosome (8, 35). Extrusion pumps similar to those described for antibiotic resistance have also been postulated as having a role in solvent tolerance (21). The 1996 study by Isken and de Bont showed that an intact proton motive force was required for solvent tolerance and suggested that this energy was harnessed by an efflux system to pump out toluene from the cell (21).

Because much of the initial damage caused by straight-chain and aromatic hydrocarbons occurs by disruption of phospholipid membranes (10, 22, 37–39, 48), the membrane structure of solvent-resistant strains warrants further study. In general, several mechanisms for decreasing membrane fluidity (due to temperature increases, solvents, etc.) have been demonstrated in bacteria. These include alteration of the polar head groups of phospholipids (26, 27, 41), increased relative percentage of saturated fatty acids leading to a decrease in membrane fluidity (19), and conversion of *cis*-unsaturated fatty acids to the *trans* isomer (15, 16, 45). *trans*-Unsaturated fatty acids are made by solvent-sensitive strains (14, 31) as well as by bacteria under starvation conditions (13), and thus their production appears to be a general stress response in some bacteria. *Pseudomonas putida* Idaho (8) grows in high concentrations of organic solvents and thus likely harbors some mechanism that either prevents or compensates for membrane damage.

In a previous study, it was observed that *P. putida* Idaho showed an increase in the amount of total membrane phospholipid fatty acid produced following exposure to xylene (33). One possible explanation for this is increased rate of membrane biosynthesis in response to exposure to xylene. An increased rate of membrane biosynthesis would be beneficial to this strain, as it would allow rapid repair of membrane damaged by exposure to xylene.

In this study, the objectives were to characterize the responses of two strains of *P. putida* to various concentrations of *o*-xylene in terms of growth and with respect to membrane adaptation in the presence and absence of monoaromatic hydrocarbons. The first goal was to determine differences in phospholipid structure and composition between *P. putida* Idaho and *P. putida* MW1200 when each strain is exposed to increasing concentrations of *o*-xylene. Previously observed changes in fatty acid concentration (33) were assigned to specific phospholipids. Following establishment of these differences, the second goal of this study was to determine the rates of membrane biosynthesis and phospholipid turnover in both solvent-tolerant and solvent-sensitive *P. putida* strains.

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TABLE 1. Mole percentages of each class of phospholipids and total phospholipid obtained from *P. putida* MW1200 and *P. putida* Idaho in the presence and absence of *o*-xylene

PL class[a]	Mol%				
	MW1200		Idaho		
	0 ppm	75 ppm	0 ppm	75 ppm	200 ppm
PE	55.97	66.14	73.93	77.07	77.40
PG	28.79	30.74	14.21	19.53	20.43
CL	11.24	6.60	8.88	1.81	0.96
PA	2.40	1.12	1.88	0.93	1.36
PS	1.56	0.78	1.07	0.63	0.55
Total PL (nmol of phospholipid/mg [dry wt] ± 1 SD)	77.0 ± 13.2	38.4 ± 10.7	84.5 ± 12.9	115.2 ± 9.81	166.4 ± 10.3

[a] PL, phospholipid; PE, phosphatidylethanolamine; PG phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; PS, phosphatidylserine.

MATERIALS AND METHODS

Strains and culture conditions. Two strains of *P. putida* were used. Both strains contain genes for the degradation of toluene on the chromosome (8, 40), and neither strain can degrade *o*-xylene. *P. putida* Idaho was first isolated by James Wolfram and Robert Rogers at Idaho National Engineering Laboratories (U.S. patent 4,863,872, September 1989). This strain withstands aqueous solutions saturated with toluene and xylenes (8). The second strain used for these experiments is *P. putida* MW1200. This strain can tolerate a maximum of 75 ppm of *o*-xylene. Exposure to concentrations greater than 75 ppm causes loss of viability (33).

Unless otherwise stated, for all experiments each strain was grown in continuous culture, using a minimal salts medium with 2.7 g of sodium succinate per liter as the carbon source. The minimal salts medium was formulated as follows: 0.7 g of K_2HPO_4, 0.7 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.3 g of $MgSO_4$, and 1 ml of Wolfe's minerals (3) per liter of nanopure water (pH 6.8). For the experiments using organic solvents, *o*-xylene was added either directly to the aqueous phase of the medium or through the feed medium of the continuous culture. The concentration of *o*-xylene in the aqueous phase was verified by gas chromatographic analysis.

Growth rates were previously established for each strain in the presence and absence of *o*-xylene (34). *P. putida* MW1200 demonstrated a doubling time of 1.4 h in both the absence and presence of 75 ppm of *o*-xylene. *P. putida* Idaho showed doubling times of 1.4 h in the absence of *o*-xylene and 1.5 h in the presence of *o*-xylene. This difference was not statistically significant.

Phospholipid extraction and identification of phospholipid class. Cells (100 ml) were removed from continuous culture (in the absence or presence of *o*-xylene) and centrifuged, the supernatant was discarded, and the cell pellet was lyophilized. The pellet was then extracted by a modified Bligh-Dyer technique (12). The organic extract containing the total lipid portion was applied to a silicic acid column. Phospholipids were eluted with methanol following chloroform and acetone washes of the column, which removed neutral lipids and glycolipids.

The polar lipid fraction obtained from silicic acid separation was further separated into phospholipid classes by one-dimensional thin-layer chromatography (TLC) (24) and developed in chloroform-methanol-water-acetic acid (85:22.5:3:15). Phospholipids were visualized with 254-nm UV light following treatment with rhodamine. Individual phospholipids were located by comparing the R_f values of the sample lane with the R_f values of known standards. Phospholipid structures were verified by direct probe mass spectrometry following elution of the phospholipid from the silica gel. The eluted phospholipids were also analyzed for fatty acid content (see below).

Phospholipids were also quantified and separated by using high-pressure liquid chromatography (HPLC) with an evaporative light-scattering detector (6). We used a binary gradient consisting of solvent A (chloroform–methanol–30% ammonium hydroxide [80:19.5:0.5]) and solvent B (chloroform–methanol–water–30% ammonium hydroxide [60:34:5.5:0.5]), with a flow rate of 1 ml/min. Use of this detector is a destructive technique that does not allow for further characterization of the phospholipids, and therefore the detector was used only for quantitation purposes.

Fatty acid analysis. Phospholipid fatty acid methyl esters were obtained by a transesterification reaction (13) and were separated by gas chromatography. Individual fatty acids were identified by comparing the retention times with those of fatty acid methyl ester standards. Individual fatty acid methyl ester peak areas were quantified by comparison to a peak area of known concentration of internal standard (dinonadecanoic phosphatidylcholine, 25 pmol/μl) added to the sample prior to extraction. Fatty acid structure was verified by mass spectrometry. Double bond positions of monounsaturated fatty acids were verified by mass spectral analysis of dimethyl disulfide adducts of the fatty acids (28).

Pulse-chase protocol. A pulse-chase methodology was used in this experiment (23, 32, 36). Each strain was grown in continuous culture with succinate mineral salts medium prior to the experiment. Plate counts were performed from a nonlabeled culture treated identically to the experimental cultures at the start and end of this experiment to estimate cell number. Two controls were used: an abiotic control, to which no cells were added; and azide-killed cells, to account for any labeled phosphate that was associated with the cell surface.

Cells (100 ml) were removed from the continuous cultures and filtered (Supor filters; 0.2-μm pore size) to remove the cells from the culture. The cells retained on the filter were washed three times in phosphate-free buffer. Washed cells were transferred to 250-ml culture flasks containing 100 ml of minimal salts medium supplemented with 10 mM succinate or minimal salts-succinate medium supplemented with 75 or 200 ppm of *o*-xylene. The controls contained only the minimal salts-succinate medium. [^{32}P]orthophosphoric acid (50 μCi) was added to each flask. The cells were exposed to the [^{32}P]orthophosphoric acid for 60 min, harvested by filtration, washed in phosphate-free buffer, and then resuspended in unlabeled medium. The cold chase was allowed to proceed for 2.0 h.

During the initial pulse, 2-ml samples were removed every 10 min for the first hour and then every 30 min until the end of the cold chase. The phospholipids were extracted in the same manner as described previously. One hundred-microliter aliquots were removed from the aqueous and organic fractions (which contained the total lipid extract) of the Bligh-Dyer extraction, and the radioactivity was assessed by scintillation counting. The organic fraction was then applied to a TLC plate to isolate the individual phospholipids (24). Radioactivity was measured directly from the TLC plate by using the Bioscan Imaging Scanner system (2). Following data acquisition from the TLC plate, all radioactive spots were removed and subjected to fatty acid analysis for quantitation purposes. Specific activity was then calculated from these data. This experiment generated total rate of incorporation of radiolabel into phospholipid, total rate of loss from phospholipid, and incorporation values and turnover data for individual phospholipids.

Statistics. Data points shown are averages of three independent experiments, with all error bars on graphs representing 1 standard deviation. Calculations were performed with GraphPad Prism software (San Diego, Calif.). Values in tables are averages of at least three experiments and are shown as means ± 1 standard deviation. *t* tests were used to determine significant differences, using $\alpha = 0.01$.

Total phospholipid biosynthesis and turnover rates were calculated by linear regression analysis. Rates of biosynthesis and turnover are the results of three independent experiments. Each data point represents the mean of three experiments. Error bars on the graphs represent 1 standard deviation. *t* tests were used to determine significant differences between the biosynthesis and turnover rates of each strain under the different growth conditions ($\alpha = 0.01$).

RESULTS

Phospholipid composition. The phospholipid composition of each strain is shown in Table 1. Initial characterization of the phospholipid composition showed that the major classes of phospholipid of both strains were phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (diphosphatidylglycerol). HPLC analysis showed the Idaho strain to have a greater percentage of phosphatidylethanolamine than *P. putida* MW1200 when grown with succinate. Phosphatidylserine and phosphatidic acid were detected in trace amounts in both strains. Following exposure to xylene, changes in phospholipid content were seen in both strains. Strain Idaho showed an overall increase in phospholipid following exposure to 75 and 200 ppm of *o*-xylene. Strain MW1200 showed a decrease in the amount of phospholipid following exposure to its maximum tolerable limit of xylene.

TABLE 2. Changes in fatty acid composition of individual phospholipids of *P. putida* Idaho following exposure to *o*-xylene[a]

Fatty acid type	Mol% (mean ± 1 SD [n = 3])									
	PE		PG		CL		PA		PS	
	−Xyl	+Xyl	−Xyl	+Xyl	−Xyl	+Xyl	−Xyl	+Xyl	−Xyl	+Xyl
Saturated	59 ± 12	60 ± 12	54 ± 9	88 ± 13	97 ± 8	95 ± 9	69 ± 7	86 ± 8	56 ± 9	59 ± 10
cis unsaturated	35 ± 6	17 ± 4	46 ± 7	9 ± 3	3 ± 0.6	5 ± 1	31 ± 6	10 ± 2	40 ± 7	13 ± 3
trans unsaturated	6 ± 0.7	23 ± 5	0	3 ± 1	0	0	0	7 ± 2	4 ± 2	28 ± 4

[a] PE, phosphatidylethanolamine; PG phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; PS, phosphatidylserine.

Analysis of the fatty acid composition of each phospholipid for each strain showed that specific changes in the fatty acid composition were localized to specific classes of phospholipid (Table 2). For both strains, the *cis-trans* isomerization occurred mainly in phosphatidylethanolamine. The increase in saturation in strain Idaho occurred mainly in phosphatidylglycerol. Strain MW1200 showed a similar but smaller increase in saturation associated with phosphatidylglycerol (not shown). Phosphatidic acid showed small changes in both saturation and levels of *cis*-unsaturated fatty acids. Cardiolipin did not show much change in fatty acid composition when it was grown under conditions of xylene saturation.

Previous analysis of the lipid A from the lipopolysaccharide (LPS) of each strain showed no compositional change for both strains grown in the presence and absence of *o*-xylene, although strain Idaho showed increased total LPS following xylene exposure (33, 34).

Time-dependent change in phospholipid content. Following observation of the increase in total phospholipid in strain Idaho following exposure to xylene, we conducted experiments to identify the time required to manifest these changes. These experiments were also run in continuous culture, which were initiated in succinate-minimal salts medium and then carried out in medium amended with 75 and 200 ppm of *o*-xylene following the time zero time point. To accomplish this, the influent lines of the continuous cultures were clamped off, and xylene was added directly to the growth chamber. The succinate-minimal salts medium was then replaced with succinate-minimal salts medium amended with either 75 ppm of *o*-xylene or 200 ppm of *o*-xylene, and the influent clamp was removed.

P. putida MW1200 showed an increase in phospholipid content at 120 h in the absence of xylene (Fig. 1A). A similar increase was seen in the presence of 75 ppm of *o*-xylene but was significantly less than the amount produced in the absence of xylene. *P. putida* MW1200 showed a decrease in phospholipid content following exposure to 200 ppm. Diglycerides were detected in the supernatant of strain MW1200 following exposure to 200 ppm of *o*-xylene at the 400-min time point, indicating cell death and hydrolysis of phospholipid (data not shown). The high variation in phospholipid content (evidenced by a high standard deviation) in both strains at the initial time points is probably due to the perturbation caused by the stop and resumption of flow in the continuous culture.

P. putida Idaho showed increases in *trans*-unsaturated fatty acids at 5 min, with maximal amounts detected at 30 min following exposure to xylene. The increase in fatty acid saturation was noted following 15 min of exposure, with the maximum amount of saturation seen at 2 h following xylene exposure. The increase in total phospholipid is observed as early as 20 min following exposure to xylene (Fig. 1B), with subsequent increases between 2 and 6 h. The same response was seen for *P. putida* MW1200 following exposure to xylene with regard to the synthesis of *trans*-unsaturated fatty acids. However, there was only a small increase in saturated fatty acids over time. Following exposure to 75 ppm of xylene, the amount of phospholipid synthesized was less than that observed for growth in the absence of the solvent. In the presence of 200 ppm of xylene, loss of phospholipid was noted over time, indicating cell death. This loss was small in the initial 2 h of exposure but accelerated soon after the 2-h time point.

Phospholipid biosynthesis and turnover. Cell numbers at the start of the experiment were 3.1 (±0.8) × 10^7 CFU/ml for *P. putida* MW1200 and 4.1 (±1.2) × 10^7 CFU/ml for *P. putida* Idaho. Ending cell numbers were 6.3 (±1.1) × 10^7 and 5.9 (±1.4) × 10^7 CFU/ml, respectively, indicating a small increase in population over the course of the experiment. Lipid biosynthesis rates were normalized to the increase in cell number over time. The azide-killed cells showed minimal uptake of radiolabel, with incorporation of an average of 0.12 cpm $nmol^{-1}$ min^{-1}. This value was subtracted from the rate calcu-

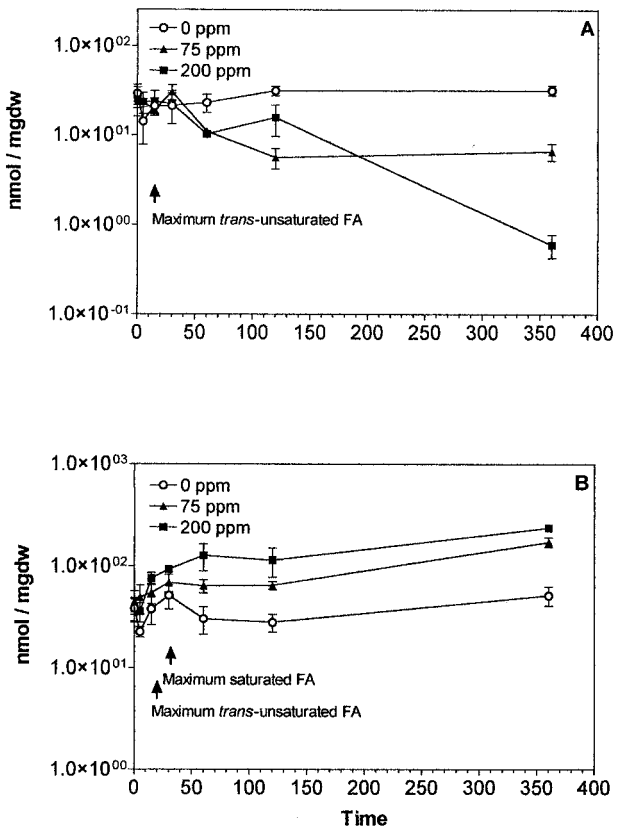

FIG. 1. (A) Change in total phospholipid concentration in *P. putida* MW1200 over time following exposure to *o*-xylene. (B) Increase in phospholipid in *P. putida* Idaho over time in the presence and absence of *o*-xylene. Phospholipid concentrations are expressed in nanomoles per milligram (dry weight) (mgdw) of cells. Error bars represent 1 standard deviation (n = 3).

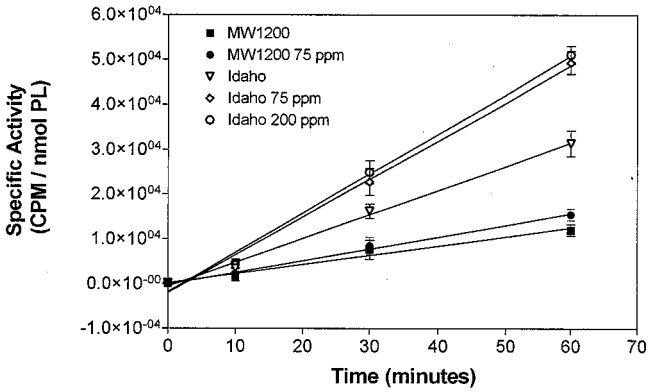

FIG. 2. Rates of [^{32}P]orthophosphate incorporation into phospholipid for *P. putida* MW1200 and *P. putida* Idaho in the absence and presence of *o*-xylene. Incorporation rates are expressed as counts per minute per nanomole of phospholipid (PL) per minute.

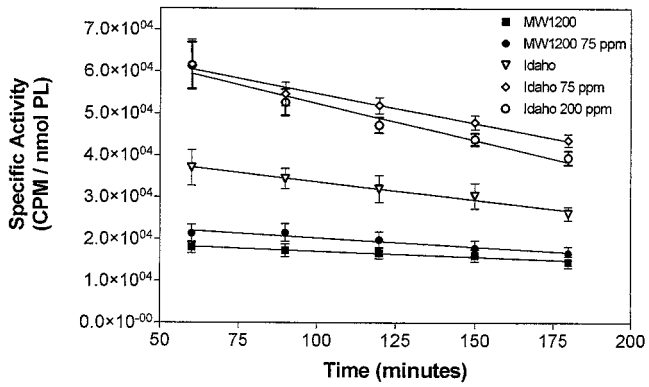

FIG. 3. Rates of [^{32}P]orthophosphate loss from phospholipid for *P. putida* MW1200 and *P. putida* Idaho in the absence and presence of *o*-xylene, expressed as in Fig. 2.

lated for each experimental strain. Incorporation of label into phospholipid was monitored over time (average of three experiments) and is expressed as specific activity in Fig. 2. Strain MW1200 showed incorporation of ^{32}P into phospholipid at an average rate of 207.3 (±21.04) cpm nmol^{-1} min^{-1} (r^2 = 0.907). In the presence of 75 ppm of *o*-xylene, this rate increased to 273.6 (±17.00) cpm nmol^{-1} min^{-1} (r^2 = 0.963). Strain Idaho showed a higher rate of incorporation. In the absence of solvent, strain Idaho exhibited an average incorporation rate of 532.7 (±23.16) cpm nmol^{-1} min^{-1} (r^2 = 0.981). In the presence of 75 ppm of *o*-xylene, this rate increased to 852.0 (±31.47) cpm nmol^{-1} min^{-1} (r^2 = 0.987). The incorporation rate for *P. putida* Idaho in the presence of 200 ppm of xylene was 882.6 (±29.23) cpm nmol^{-1} min^{-1} (r^2 = 0.989). All incorporation rates were significantly linear.

t tests (Table 3) run on the foregoing data showed the following results: (i) for strain MW1200, there was no significant difference between the rates of incorporation of radiolabel into phospholipid in cells grown in the presence and absence of xylene; (ii) there was no significant difference between the rates of incorporation of *P. putida* Idaho grown in the presence of 75 ppm of *o*-xylene and 200 ppm of *o*-xylene; (iii) there was a significant difference between the rates of incorporation of radiolabel into phospholipid in *P. putida* Idaho cells grown in the presence and absence of xylene; (iv) there was a significant difference between the rates of incorporation of radiolabel into phospholipid of *P. putida* MW1200 and *P. putida* Idaho grown in the absence of *o*-xylene; and (v) there was a significant difference between the rates of incorporation of radiolabel into phospholipid of *P. putida* MW1200 and *P. putida* Idaho grown in the presence of *o*-xylene.

Data on the loss of label (turnover) from the phospholipid are shown in Fig. 3. The rate of loss of label from strain MW1200 in the absence of *o*-xylene was 24.89 (±9.24) cpm nmol^{-1} min^{-1} (r^2 = 0.358). The rate of loss of label from the MW1200 strain in the presence of *o*-xylene was 42.89 (±11.24) cpm nmol^{-1} min^{-1} (r^2 = 0.5284). The rate of loss of label from the Idaho strain in the absence of *o*-xylene was 56.44 (±16.32) cpm nmol^{-1} min^{-1} (r^2 = 0.4793). The rate of loss of label from the Idaho strain in the presence of 75 ppm of *o*-xylene was 131.7 (±12.38) cpm nmol^{-1} min^{-1} (r^2 = 0.897). The rate of loss of label from strain Idaho in the presence of 200 ppm of *o*-xylene was 178.3 (±18.22) cpm nmol^{-1} min^{-1} (r^2 = 0.881). The rates of loss from both strains in the absence of *o*-xylene were not significantly different from zero. The rates of loss for both strains following exposure to xylene were significantly different from zero.

t tests (Table 4) run on the total phospholipid turnover data showed the following results: (i) for strain MW1200, there was no significant difference between the rates of loss of radiolabel into phospholipid in cells grown in the presence and absence of xylene; (ii) there was no significant difference between the rates of loss of *P. putida* Idaho and *P. putida* MW1200 grown in the absence of *o*-xylene; (iii) there was no significant difference between the rates of loss of *P. putida* Idaho grown in the presence of 75 ppm of *o*-xylene and 200 ppm of *o*-xylene; (iv) there was a significant difference between the rates of incorporation of radiolabel into phospholipid in *P. putida* Idaho cells grown in the presence and absence of xylene; (v) there was a significant difference between the rates of incorporation of radiolabel into phospholipid of *P. putida* MW1200 and *P. putida* Idaho grown in the presence of *o*-xylene.

TABLE 3. *t* tests for phospholipid biosynthetic rate data (α = 0.01)

Rate comparison[a]	*P* value	Significance
$MW1200_s$ vs $MW1200_{75\times}$	0.0512	Not significant
$Idaho_s$ vs $Idaho_{75\times}$	0.0006	Significant
$Idaho_s$ vs $Idaho_{200\times}$	0.0004	Significant
$Idaho_{75\times}$ vs $Idaho_{200\times}$	0.4596	Not significant
$MW1200_s$ vs $Idaho_s$	<0.0001	Significant
$MW1200_{75\times}$ vs $Idaho_{75\times}$	<0.0001	Significant
$MW1200_{75\times}$ vs $Idaho_{200\times}$	<0.0001	Significant

[a] Subscripts: s, growth in succinate-minimal salts medium; 75×, growth in succinate-minimal salts medium plus 75 ppm of *o*-xylene; 200×, growth in succinate-minimal salts medium plus 200 ppm of *o*-xylene.

TABLE 4. *t* tests for rate of loss (phospholipid turnover) data (α = 0.01)

Rate comparison[a]	*P* value	Significance
$MW1200_s$ vs $MW1200_{75\times}$	0.0242	Not significant
$Idaho_s$ vs $Idaho_{75\times}$	0.0014	Significant
$Idaho_s$ vs $Idaho_{200\times}$	0.0002	Significant
$Idaho_{75\times}$ vs $Idaho_{200\times}$	0.8452	Not significant
$MW1200_s$ vs $Idaho_s$	0.0186	Not significant
$MW1200_{75\times}$ vs $Idaho_{75\times}$	0.0014	Significant
$MW1200_{75\times}$ vs $Idaho_{200\times}$	0.0002	Significant

[a] Subscripts: s, growth in succinate-minimal salts medium; 75×, growth in succinate-minimal salts medium plus 75 ppm of *o*-xylene; 200×, growth in succinate-minimal salts medium plus 200 ppm of *o*-xylene.

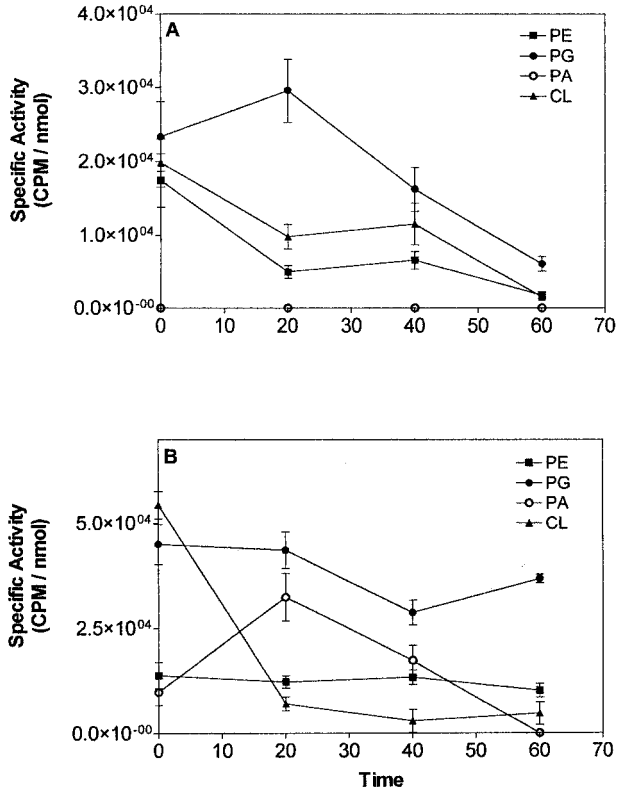

FIG. 4. (A) Turnover of individual phospholipids of *P. putida* MW1200 in the absence of *o*-xylene. (B) Turnover of individual phospholipids of *P. putida* MW1200 in the presence of 75 ppm of *o*-xylene. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin. Error bars represent 1 standard deviation ($n = 3$).

In the absence of solvent, strain MW1200 showed greatest incorporation of label into phosphatidylglycerol, followed by cardiolipin and phosphatidylethanolamine. Cardiolipin showed the most rapid turnover, followed by phosphatidylglycerol and phosphatidylethanolamine in *P. putida* MW1200 (Fig. 4A). Phosphatidic acid was detected only at very low levels, and phosphatidylserine was not detected at all. In the presence of *o*-xylene, cardiolipin showed the greatest incorporation of label, followed by phosphatidylglycerol, phosphatidic acid, and phosphatidylethanolamine (Fig. 4B). Cardiolipin again showed the most rapid turnover, followed by phosphatidic acid, a phosphatidylglycerol precursor. Phosphatidylethanolamine and phosphatidylglycerol showed relatively little turnover following exposure to *o*-xylene.

In the absence of *o*-xylene, *P. putida* Idaho exhibited the greatest incorporation of label into phosphatidylglycerol, followed by phosphatidylethanolamine and cardiolipin (Fig. 5A). Phosphatidylglycerol was the most rapidly turned over phospholipid, followed by phosphatidylethanolamine and cardiolipin in *P. putida* Idaho. Phosphatidic acid was detected only at low levels, with no phosphatidylserine detected. In the presence of 75 ppm of *o*-xylene, phosphatidylglycerol showed the greatest incorporation of label, followed by phosphatidic acid, phosphatidylethanolamine, and cardiolipin. Phosphatidic acid showed the greatest turnover, followed by phosphatidylglycerol (Fig. 5B). Phosphatidylethanolamine and cardiolipin both showed turnover, but at a much lower rate. In the presence of 200 ppm of *o*-xylene, phosphatidylethanolamine showed the greatest incorporation of label, followed closely by phosphatidylglycerol (Fig. 5C). Cardiolipin and phosphatidic acid also showed incorporation of label. Interestingly, at this concentration, phosphatidylethanolamine showed the greatest turnover, followed by cardiolipin, phosphatidylglycerol, and phosphatidic acid.

DISCUSSION

The two *P. putida* strains were similar in phospholipid composition, harboring typical phospholipids of fluorescent pseudomonads (47). Following exposure to xylene, strain MW1200

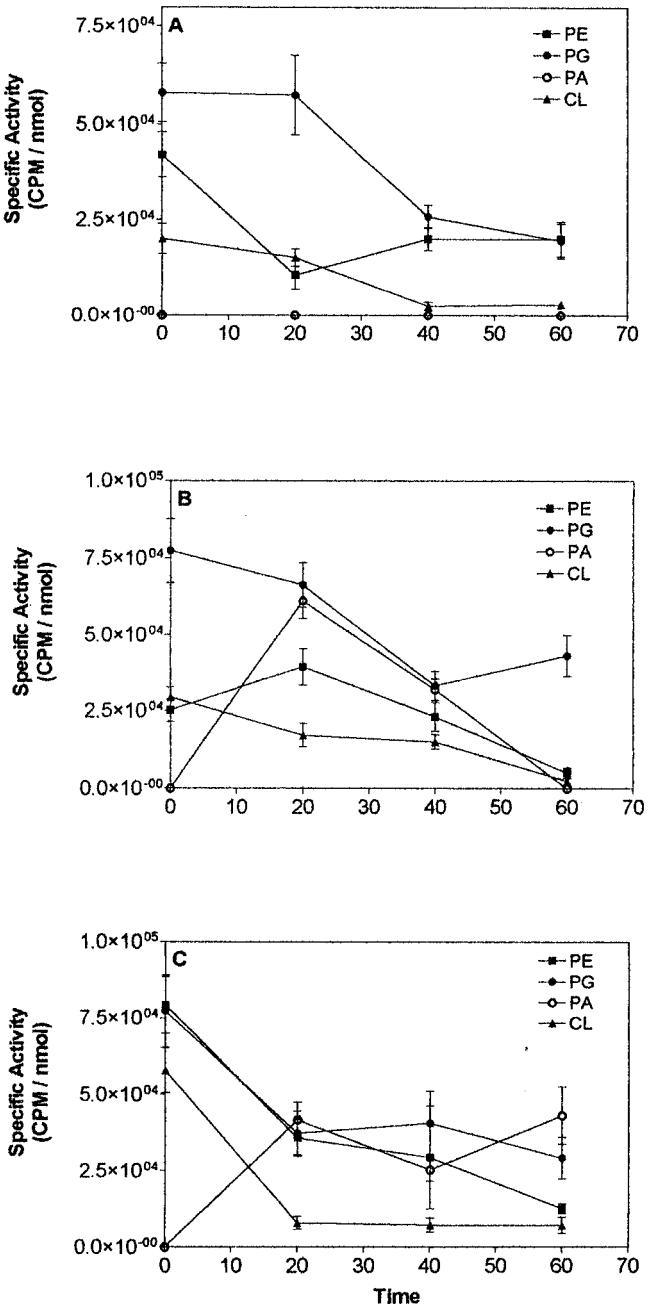

FIG. 5. (A) Turnover of individual phospholipids of *P. putida* Idaho in the absence of *o*-xylene. (B) Turnover of individual phospholipids of *P. putida* Idaho in the presence of 75 ppm of *o*-xylene. (C) Turnover of individual phospholipids of *P. putida* Idaho in the presence of 200 ppm of *o*-xylene. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin. Error bars represent 1 standard deviation ($n = 3$).

showed a decrease in all phospholipid classes, consistent with data presented by Weber and deBont (44). With *o*-xylene exposure, strain Idaho consistently showed greater amounts of phosphatidylethanolamine than strain MW1200. Phosphatidylethanolamine has a higher melting point than phosphatidylglycerol, and so increased proportions may help counter increased membrane fluidity in the xylene-exposed cells (27). An increase in phosphatidylethanolamine to counter the effects of solvents is not a common response among bacteria (44). Following exposure to organic solvents, decreases in the proportion of phosphatidylethanolamine were observed in *Escherichia coli* (18, 19), *Mycobacterium smegmatis* (43), *P. putida* S12 (44, 45), *P. putida* MW1200 (this study), and *Zymomonas mobilis* (5, 6). *P. putida* Idaho and *Microsporon* sp. are the only bacteria that exhibit increased levels of phosphatidylethanolamine (this study and reference 4). The increase in phosphatidylglycerol was a more common response, exhibited by *P. putida* S12, a solvent-tolerant organism, *P. putida* Idaho, *E. coli*, and the archaebacterium *Acholeplasma laidlawii* (46). Most of these organisms also showed an increase in cardiolipin (diphosphatidylglycerol), a response not shared by *P. putida* Idaho.

The total phospholipid content of strain MW1200 decreased by 70% following exposure to xylene, a response shared by *M. smegmatis* and *E. coli* (18, 19, 43). Because of the mechanism of action of xylene, it is likely that the loss resulted from extraction of the membrane components to the surrounding medium. In contrast, *P. putida* Idaho increased its total phospholipid by 100% (Table 1; Fig. 1B), which suggests that a membrane repair mechanism may be present in strain Idaho that allows for replacement of damaged membrane components.

Although *P. putida* MW1200 showed a slight increase in the mean phospholipid biosynthetic rate following exposure to *o*-xylene, it did not exhibit increased phospholipid following exposure to xylene. On the contrary, it showed a loss of phospholipid following exposure to *o*-xylene (Table 1; Fig. 1A). Therefore, it seems that a small increase in biosynthetic rate of phospholipid was not sufficient to maintain membrane integrity following exposure to solvents.

P. putida Idaho showed a basal rate of phospholipid biosynthesis that was significantly greater than that of *P. putida* MW1200. Interestingly, in the absence of *o*-xylene, it did not exhibit significantly greater total phospholipid than the solvent-sensitive strain ($P = 0.2981$). In the presence of *o*-xylene, the rate of phospholipid biosynthesis increased significantly. The fact that the biosynthetic rate was significantly greater following exposure to *o*-xylene than in the absence of xylene indicates that the increased rate is an inducible event.

Although this response has not yet been reported in the literature as a response to solvents, increased phospholipid biosynthesis has been reported for other environmental stresses. For example, Russell et al. (36) reported an increase in the rate of phospholipid (phosphatidylglycerol and phosphatidylethanolamine) synthesis after shifting a *Vibrio costicola* culture to a 3 M NaCl culture from a 1 M Nacl culture. A much greater increase in phosphatidylglycerol was noted for this strain. This was thought to occur because phosphatidylglycerol is a negatively charged phospholipid, while phosphatidylethanolamine is a neutral lipid. This increase in charged phospholipids was thought to be important in maintaining charge balance at the membrane level. To accommodate overexpressed membrane-bound *alk* gene products, *E. coli* demonstrated significantly increased membrane phospholipid (29).

The rate of total phospholipid turnover in both strains was higher in xylene-exposed cells than in cells not exposed to xylene. Membrane phospholipids damaged by xylene must be replaced to maintain membrane integrity. The greater rate of turnover in strain Idaho indicates that this strain may be more efficient at scavenging damaged membrane components for use in phospholipid synthesis.

Analysis of individual phospholipid turnover showed that phosphatidylethanolamine was the most abundant phospholipid in both strains. Therefore, it seems likely that the most incorporation of labeled phosphate would be in phosphatidylethanolamine. However, in strain MW1200, it showed the lowest incorporation and turnover in both the presence and absence of *o*-xylene. In strain Idaho, phosphatidylethanolamine also showed the least incorporation and turnover in the absence of *o*-xylene and in the presence of 75 ppm of *o*-xylene. Only at 200 ppm did phosphatidylethanolamine show significant turnover. It is possible that phosphatidylethanolamine does not incur as much damage as other phospholipids and therefore does not need to be synthesized at as a high a level as the other phospholipids. This may explain why incorporation was high in strain Idaho at 200 ppm and not at 75 ppm.

Interestingly, cardiolipin showed significant turnover in both strains grown in the presence of xylene. This was surprising because cardiolipin constitutes a very low proportion of the total phospholipid in these *P. putida* strains. Increases in phosphatidic acid and phosphatidylglycerol followed decreases in cardiolipin specific activity, indicating that phosphatidylglycerol is synthesized at the expense of cardiolipin. The fact that the amount of cardiolipin proportionally decreases in xylene-exposed cells (in both strains) supports this conclusion.

In both strains, phosphatidylglycerol generally showed the greatest incorporation of label. It also showed a higher rate of turnover than phosphatidylethanolamine in all cases except *P. putida* Idaho in the presence of 200 ppm of *o*-xylene. Although this is the second most abundant phospholipid in each strain, it accounts for only 25% or less of the total phospholipid in both strains. Because it shows high incorporation of label and significant turnover, it is likely that this phospholipid incurs more damage by xylene than phosphatidylethanolamine and needs to be replaced more rapidly. This could also account for the rapid turnover and proportional decrease in cardiolipin. The turnover of cardiolipin, which contains two molecules of phosphatidylglycerol, is an efficient method to replenish phosphatidylglycerol pools.

It is apparent that the solvent-tolerant strain studied here has a statistically significant increased biosynthetic rate following exposure to *o*-xylene. This indicates that an inducible membrane repair mechanism which allows rapid repair of damaged membrane components exists in *P. putida* Idaho. Investigators of solvent-tolerant organisms have noted an increased requirement for magnesium for these strains. Ramos et al. (35) and Inoue et al. (20) showed that the addition of excess magnesium ions aided in decreasing the lag phase for cells grown in high concentrations of toluene. If rapid membrane repair occurs in the solvent-tolerant strains, then a requirement for magnesium ions would exist because magnesium is known to be one of the important cations in phospholipid and LPS stabilization (25).

While an increased rate of membrane biosynthesis in response to cell damage has not been previously reported, rapid repair of damaged cell components as a resistance mechanism is not an unusual phenomenon. *Deinococcus radiodurans*, for example, has a DNA polymerase that is 100 to 150 times as efficient as that found in *E. coli*. This attribute allows the species to survive high doses of ionizing radiation (42). Resistance to the antibiotic trimethoprim has been acquired by some bacterial strains by overproducing the target molecule of the antibiotic, dihydrofolate reductase, by 600 times the nor-

mal level, thus negating the effect of trimethoprim. In *Staphylococcus aureus*, resistance to methicillin is acquired through overproduction of PBP2a (11). In *E. coli*, a mutation in cardiolipin synthesis conferred resistance to 3,4-dihydroxybutyl 1-phosphonate (17).

Although many investigators have examined fatty acid content and phospholipid content of various bacterial strains in response to organic solvents, to date no one has examined phospholipid biosynthetic rates following exposure to organic solvents. It has been shown that *P. putida* Idaho demonstrates an increased rate of phospholipid biosynthesis following exposure to the organic solvent *o*-xylene, a response not shown in a similar but solvent-sensitive strain, *P. putida* MW1200. This energy-dependent process promotes repair of membranes damaged by solvents and appears to enhance tolerance to high concentrations of organic solvents in *P. putida* Idaho.

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