Phosphorus-31 Nuclear Magnetic Resonance Study of the Effect of Pentachlorophenol (PCP) on the Physiologies of PCP-Degrading Microorganisms

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Free and agarose-encapsulated pentachlorophenol (PCP)-degrading *Sphingomonas* sp. isolates UG25 and UG30 were compared to *Sphingomonas chlorophenolica* ATCC 39723 with respect to the ability to degrade PCP. Pretreatment of the UG25 and UG30 strains with 50 μ g of PCP per ml enabled the cells to subsequently degrade higher levels of this environmental pollutant. Similar treatment of ATCC 39723 cells had no effect on the level of PCP degraded by this strain. Phosphorus-31 nuclear magnetic resonance spectra of agarose-immobilized strains UG25 and UG30 grown in the absence of PCP showed that there was marked deenergization of the cells upon exposure to a nonlethal concentration of PCP (120 μ g/ml). For example, no transmembrane pH gradient was observed, and the ATP levels were lower than the levels obtained in the absence of PCP. The transmembrane pH gradient and ATP levels were restored once the immobilized cells had almost completely degraded the PCP in the perfusion medium. PCP-pretreated cells, on the other hand, maintained their transmembrane pH gradient and ATP levels even in the presence of high levels of PCP. The ability of PCP-pretreated strain UG25 and UG30 cells to remain energized in the presence of PCP was shown to correlate with an altered membrane phospholipid profile; these cells had a higher concentration of cardiolipin than cells cultured in the absence of PCP. Strain ATCC 39723, which did not degrade higher levels of PCP after PCP pretreatment, did not show this response.

Pentachlorophenol (PCP) is a polychlorinated aromatic compound that is primarily used as a wood preservative in the lumber industry in North America. Other roles for PCP include use as an herbicide, an insecticide, and a fungicide (22). The high toxicity of PCP makes cleanup of contaminated soil necessary, and bioremediation is an attractive alternative to conventional physical and chemical methods of soil reclamation (1, 22).

PCP is degraded by a number of microorganisms, including the well-studied strain *Flavobacterium chlorophenolica* ATCC 39723 (27). This strain has been reclassified as a member of the genus *Sphingomonas* (10). In *Sphingomonas chlorophenolica* ATCC 39723, PCP first undergoes oxygenolytic dechlorination to tetrachloro-*p*-hydroquinone by a type 4 monooxygenase (35), and then tetrachloro-*p*-hydroquinone is further reductively dechlorinated to 2,6-dichloro-*p*-hydroquinone by a dehalogenase belonging to the theta class of the glutathione *S*-transferase superfamily (23). A third enzyme, first characterized as a chlorohydrolase (17) but later shown to be an Fe²⁺-requiring dioxygenase (34), cleaves the ring of 2,6-dichloro-*p*-hydroquinone at an undetermined position. The resulting product is further metabolized to nontoxic end products. The evolution of this three-enzyme pathway consisting of a PCP-inducible type

4 monooxygenase (PcpB), a constitutively expressed dehalogenase (PcpC), and a PCP-inducible dioxygenase (PcpA) has recently been reviewed (6).

We previously isolated and characterized two bacterial strains from PCP-contaminated soil samples (18). These isolates, *Pseudomonas* sp. strains UG25 and UG30, were subsequently also reclassified as members of the genus *Sphingomonas* based on lipid analysis and fatty acid profiles (19). By using DNA hybridization techniques, the UG25 and UG30 isolates were shown to possess genes homologous to *pcpB* and *pcpC* in strain ATCC 39723, suggesting that similar pathways for PCP degradation are present in these three *Sphingomonas* strains (18). This suggestion is supported by our recent success in cloning, sequencing, and expressing all three gene products, including that of the *pcpA* gene (unpublished data). The UG25 and UG30 genes exhibit about 90% sequence identity with the ATCC 39723 genes.

In an initial study we determined long-term PCP mineralization thresholds for strains UG25 and UG30. Using phosphorus-31 nuclear magnetic resonance (³¹P NMR) spectroscopy, we also observed that addition of PCP to concentrated cell suspensions resulted in collapse of the transmembrane pH gradient and lower nucleoside triphosphate levels (18). However, ³¹P NMR data obtained with *S. chlorophenolica* ATCC 39723 showed that cells induced with 50 μ g of PCP per ml during the early exponential phase were resistant to the uncoupling action of PCP (29). In the present study we extended our previous work by investigating how PCP pretreatment affected the physiology of strains UG25 and UG30. Batch

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culture mineralization studies using either cell suspensions or agarose-immobilized cells were performed with all three *Sphingomonas* strains (UG25, UG30, and ATCC 39723) to examine how pretreatment with 50 μ g of PCP per ml affects mineralization thresholds. Agarose-immobilized cells were subsequently used in long-term ^{31}P NMR perfusion studies in which the effects of PCP on the cellular transmembrane pH gradient and ATP levels were monitored over time. We also compared the lipid fractions of cells grown in the absence and in the presence of PCP to determine the effect of PCP on the cellular membrane composition. Finally, the effects of PCP on oxygen consumption levels in resting and glutamate-metabolizing cell suspensions were studied in an attempt to determine whether PCP is an uncoupler or inhibitor of oxidative phosphorylation in strains UG25 and UG30.

MATERIALS AND METHODS

Chemicals and media. Minimal salt glutamate (MSG) medium contained (per liter) 0.5 g of NaNO_3 , 1.65 g of K_2HPO_4 , 0.17 g of KH_2PO_4 , 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 4 g of monosodium glutamate. The final pH was 7.3. HEPES-buffered low-phosphate MSG medium (HMSG) contained 50 mM HEPES (pH 7.3), 0.165 g of K_2HPO_4 per liter, and 0.017 g of KH_2PO_4 per liter; the other ingredients were the same as those in MSG medium. MS medium and HMS medium were variations of MSG medium and HMSG medium, respectively, but contained no glutamate.

Sodium pentachlorophenolate (PCP) (Fisher Scientific) was prepared as 500-mg/liter stock solutions in either MSG or HMSG medium. After autoclaving, the solutions were stored in the dark to prevent photodegradation. Prior to use the concentration of PCP was calibrated by reading the absorbance at 318 nm using a molar extinction coefficient of 3,863.

Microorganisms and growth conditions. PCP-degrading *Sphingomonas* sp. strains UG25 and UG30 and *S. chlorophenolica* ATCC 39723 were transferred weekly to fresh MSG medium plates (MSG medium plus 15 g of agar per liter). The plates were incubated at 30°C for 2 to 3 days before they were stored at 22°C. The strains were cultured in MSG medium at 30°C with shaking at 300 rpm (see below).

PCP degradation studies with cell suspensions. Aliquots (25 ml) of mid-exponential-phase cells (optical density at 600 nm [OD_{600}], 0.5; 8×10^7 CFU/ml) grown in MSG medium were diluted with equal volumes of fresh MSG medium containing PCP so that the final PCP concentrations varied from 0 to 350 μ g/ml. Portions (50 ml) of the resulting cultures (2×10^9 cells per experiment) were dispensed into sterile 250-ml Erlenmeyer flasks and incubated at 30°C with shaking at 300 rpm. Samples of cultures were monitored for cell growth by monitoring changes in the OD_{600} , and then aliquots were each diluted with an equal volume of 1.0 N NaOH and centrifuged at 13,000 rpm in a desk top Microfuge for 2.5 min. The absorbance at 318 nm of each resulting supernatant was monitored to determine the PCP concentration. For PCP-induced cells, the protocol described above was used, except that mid-exponential-phase cultures were exposed to 50 μ g of PCP per ml for 2 to 3 h before they were transferred to fresh sterile media containing PCP at the concentrations indicated above. Experiments were repeated three times, and each time a freshly prepared inoculum of cells was used.

PCP degradation studies with agarose-immobilized cells. All procedures were performed aseptically. Mid-exponential-phase cells were cooled to 4°C on ice, harvested by centrifugation, and resuspended in 20 ml of sterile HMS medium. The resulting cell suspension was subsequently mixed at 37°C with an equal volume of 4% low-temperature-gelling agarose (type VII; Sigma). Beads were generated in sterile vegetable oil as previously described (20). After cooling, the agarose beads were sized with wire nets. Beads between 0.5 and 1.0 mm in diameter were then washed so they were free of oil. For each experiment 3-g (wet weight) portions of beads containing approximately 2×10^9 cells were transferred to 250-ml Erlenmeyer flasks containing 50 ml of MSG medium supplemented with PCP at concentrations ranging from 0 to 450 μ g/ml. The flasks were incubated and analyzed for cell growth and PCP degradation as described above. Less than 10% free cells were observed in the medium after 24 h. For studies involving PCP-pretreated immobilized cells, the beads were first incubated for 2 h in MSG medium containing 50 μ g of PCP per ml. After this the cells were sieved free of medium, cultured, and incubated as described above. These ex-

periments were repeated three times, and each time a new preparation of immobilized cells was used.

Preparation of immobilized cells for NMR. The protocol used to prepare immobilized cells for NMR was essentially the same as that described above, except that freshly immobilized cells were incubated for 18 h in HMSG medium to increase the cell density in the beads. After this incubation period the beads were drained, and a 10-g sample was transferred aseptically to a 15-mm NMR tube. A sealed capillary containing 0.2 M methylenediphosphonic acid (MDP) was included as a chemical shift and intensity reference standard. A modified Teflon plug allowed incoming, oxygenated medium to enter at the bottom of the NMR tube and the exiting medium to be siphoned from the top of the beads. The beads were perfused for 12 h at a rate of 5 ml/min from a reservoir containing 250 ml of HMSG medium. This medium was bubbled with oxygen. After 12 h the medium in the reservoir was replaced with fresh HMSG medium containing 120 μ g of PCP per ml (perfusion using nontreated cells) or 175 μ g of PCP per ml (perfusion using PCP-pretreated cells). Incubation was continued for another 30 to 50 h. During this time aliquots of the medium were periodically withdrawn so that PCP degradation could be monitored as described above. Experiments were performed in duplicate with strain UG25 and with strain UG30.

Preparation of detergent-phospholipid micelles for NMR analysis. Cells were grown to an OD_{600} of 1.0 in MSG medium or MSG medium containing 50 μ g of PCP per ml. In the latter case, as PCP was degraded, fresh aliquots of PCP were periodically added to keep the level in the medium between 30 and 50 μ g/ml. Cells were harvested by centrifugation and washed once in HMSG medium. Membrane vesicles were prepared by the method of Kaback (14). The samples used for NMR (total volume, 6 ml) contained 35 mg of vesicles per ml, 0.32% sodium deoxycholate, 3 mM EDTA, 0.35 mM MDP, and 8.3% D_2O in 50 mM Tris buffer (pH 8.4). Under these conditions deoxycholate generated micelles which had a much narrower NMR line width than vesicles. D_2O was used for lock purposes, and MDP served as an internal standard.

Extraction of cell lipids for acyl chain analysis. Cells grown under the conditions described above were harvested and immediately extracted with chloroform-methanol- H_2O as described by Bligh and Dyer (2). The lipid fraction was dried under nitrogen gas in ampoules, sealed, and sent to Microbial ID, Inc. (Newark, Del.) for commercial analysis of the fatty acid composition.

NMR conditions. ^{31}P NMR spectra were obtained at 25°C with a Bruker AM-400 wide-bore spectrometer by using 15-mm NMR tubes. The parameters used to acquire spectra of the immobilized cells were the parameters described by Lohmeier-Vogel et al. (20). The parameters used to study the detergent-phospholipid micelles were the parameters used previously for analysis of yeast cell extracts (21), except that the recycle time was 5.1 s. All experiments were conducted a minimum of two times.

Oxygen uptake experiments. *Sphingomonas* sp. strain UG30 was grown to an OD_{600} of 1.0 in 1 liter of MSG medium, harvested by centrifugation, washed once with sterile MS medium (pH 7.4), and resuspended in 100 ml of MS medium. The cells were incubated at 30°C with shaking at 200 rpm for 2 h to starve them of endogenous carbohydrate stores. After this the cells were pelleted and then resuspended in fresh MS medium at an OD_{600} of 1.0. Oxygen consumption by resting cells was measured at 22°C with an oxygen electrode (YSI 5739 D.O. probe; Yellow Springs Instrument Co.). The substrate glutamate (final concentration, 4 g/liter) and/or the substrate sodium PCP (0 to 100 μ g/ml) was added 10 min after oxygen measurement commenced.

RESULTS

Determination of PCP degradation thresholds. Figure 1 summarizes the PCP degradation thresholds for freely suspended or agarose-immobilized *Sphingomonas* sp. strains UG25 and UG30 and *S. chlorophenolica* ATCC 39723. In this study the PCP degradation threshold was defined as the level of PCP which cells could degrade by 50% during incubation for 24 h. Occasionally, the PCP in a culture containing 100 μ g of PCP per ml was completely degraded by the cells but no degradation was observed in a culture containing 150 μ g of PCP per ml. In such cases we estimated that the degradation threshold was the average of the two values.

The data in Fig. 1 show that *Sphingomonas* sp. strains UG25 and UG30 had PCP degradation thresholds that were around 50% lower than that observed with strain ATCC 39723. After

FIG. 1. PCP thresholds for batch cultures of *Sphingomonas* sp. strains UG30 and UG25 and *S. chlorophenolica* ATCC 39723. Solid bars, suspension cultures; cross-hatched bars, suspension cultures of cells pretreated with 50 µg of PCP per ml; gray bars, immobilized cultures; open bars, immobilized cultures pretreated with 50 µg of PCP per ml.

PCP pretreatment, however, strains UG25 and UG30 exhibited statistically significant 30 to 50% increases in their PCP degradation thresholds. *S. chlorophenolica* ATCC 39723, on the other hand, did not appear to benefit from PCP pretreatment. When all three strains were immobilized in agarose beads, however, they exhibited 10 to 20% increases in their PCP degradation thresholds compared to suspension cultures.

³¹P NMR studies with immobilized UG25 and UG30. Figure 2A to E show the collapse and recovery of the pH gradient and ATP levels as a function of time when immobilized UG30 cells were perfused with HMSG medium containing 120 µg of PCP per ml. The spectrum in Fig. 2A, obtained in the absence of PCP, has three peaks, which were derived mainly from the α, β, and γ phosphates of ATP (and other nucleoside triphosphates). The α and β phosphates from nucleoside diphosphates like ADP have the same chemical shift as the ATP α and γ peaks, and so only the ATP β peak at −18.2 ppm most accurately reflects the intracellular ATP content. Since this peak is well resolved in the spectrum in Fig. 2A, we concluded that the cells were energized when they were perfused with oxygenated HMSG medium.

In the downfield region of the spectrum in Fig. 2A are two inorganic phosphate peaks. The smaller peak is the intracellular inorganic phosphate [$P_{i(int)}$] peak, and the larger peak is the extracellular inorganic phosphate [$P_{i(ext)}$] peak in the HMSG perfusion medium. The chemical shift of inorganic phosphate is pH sensitive and can be used to estimate the intracellular pH of living cells noninvasively (11). The chemical shift of the $P_{i(int)}$ resonance correlated with an intracellular pH of 7.8, whereas the $P_{i(ext)}$ chemical shift correlated with a pH of 7.3. The presence of two P_i peaks means that the cells had enough energy to maintain a transmembrane pH gradient.

For the spectrum in Fig. 2B the cells were exposed to perfusion medium containing 120 µg of PCP per ml. It is evident that the ATP β peak in this spectrum is smaller than the peak in the spectrum in Fig. 2A. In addition, the transmembrane pH gradient is not visible in the spectrum in Fig. 2B. We assume that this is because the proton gradient was disrupted by PCP. There is some indication that there is a reemerging ATP β peak in the spectrum in Fig. 2D, but full recovery of the transmembrane pH gradient was observed only in the spectrum in Fig. 2E.

The spectra in Fig. 2 are composed of 10 individual 1-h spectra which were averaged to obtain a better signal-to-noise ratio. Figure 3 shows the PCP concentrations that remained in the perfusion medium during these experiments. When Fig. 2 and 3 are compared, it is clear that the PCP concentration had to be 15 µg/ml or lower to allow cells to fully recover their transmembrane pH gradient and ATP levels, as was shown in the spectrum in Fig. 2E.

Figure 2F to J show the results obtained when immobilized UG30 cells were first pretreated with 50 µg of PCP per ml and then analyzed by ³¹P NMR spectroscopy. The spectrum in Fig. 2F, obtained when cells were perfused with HMSG medium in the absence of PCP, shows the expected profile for an energized cell: a distinct transmembrane pH gradient and high ATP levels. The perfusion medium was subsequently changed to HMSG medium containing 175 µg of PCP per ml. We expected to see completely deenergized cells in the spectrum in Fig. 2G, for which the average PCP concentration was 143 µg/ml, but observed only a partial collapse of the transmembrane pH gradient and some decrease in the ATP β peak. Full recovery of these parameters is apparent in the spectrum in Fig. 2H, for which the PCP levels were 80 µg/ml on average. Results virtually identical to those shown in Fig. 2 were also obtained for strain UG25 (data not shown).

Membrane phospholipid composition of PCP-degrading strains. ³¹P NMR spectra of membrane phospholipids in deoxycholate vesicles were obtained for *Sphingomonas* sp. strain UG30 and *S. chlorophenolica* ATCC 39723 (Fig. 4). The phospholipid profiles in Fig. 4A and C are profiles for cells which had been grown in the absence of PCP and the phospholipid profiles in Fig. 4B and D are profiles for cells that had been pretreated with 50 µg of PCP per ml. Most peaks in the spectra were identified by using commercially available standards including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Since upfield shifts in phospholipid families have been reported to occur as a result of acyl chain saturation (24), we also included PC, PG, and PE standards with disaturated or diunsaturated acyl chains. Some peaks in Fig. 4 were not identified and may correspond to members of the sphingolipid family. Our chemical shift values (Table 1) are similar to previously reported values for phospholipids in nondetergent solutions (26) but on average are about 0.68 ppm downfield from the values obtained in another study performed with detergent-phospholipid micelles (9) (Table 1). Because we used sodium deoxycholate rather than potassium cholate to solubilize our lipids and the pH of our samples was 8.4 rather than 7, these systematic differences were expected.

A comparison of the phospholipid profiles of PCP-pretreated and untreated *S. chlorophenolica* ATCC 39723 showed that the levels of PG, CL, PE, and PC were essentially the same (Fig. 4). In the case of strain UG30, however, a large increase in the CL component was observed when cells were grown in the presence of PCP, and there was also a small decrease in an as-yet-unidentified peak slightly upfield from PE. Identical changes in the phospholipid profile were observed for strain UG25 (data not shown). It was observed that the phospholipid composition of strain UG30 is more complex than that of ATCC 39723, which probably reflects strain differences.

FIG. 2. (A to E) ^{31}P NMR spectra for agarose-immobilized *Sphingomonas* sp. strain UG30 perfused with oxygenated HMSG medium. Each spectrum represents a 10-h period. (A) No PCP in perfusion medium (0 to 9 h). (B) Addition of 120 μg of PCP per ml to perfusion medium at 10 h (10 to 19 h). (C to E) Continuation of PCP degradation with time (20 to 49 h). Peak assignments: SP, sugar phosphomonoesters; $\text{P}_{i(\text{int})}$ and $\text{P}_{i(\text{ext})}$, intracellular and extracellular inorganic phosphate, respectively; PDE, phosphodiester from lipids and cell wall components; ATP- γ , contributions from nucleotide triphosphate γ resonances and nucleotide diphosphate β resonances; ATP- α , contributions from nucleotide triphosphate α resonances and nucleotide diphosphate α resonances; NAD and NADP resonances (reduced and oxidized); UDPG, uridine diphosphoglucose resonance; ATP- β , contributions from only the nucleoside triphosphate β resonances. (F to J) ^{31}P NMR spectra of agarose-immobilized *Sphingomonas* sp. strain UG30 pretreated for 2 h with 50 μg of PCP per ml and subsequently perfused with oxygenated HMSG medium. (F) No PCP in perfusion medium. (G) Addition of 175 μg of PCP per ml to perfusion medium. (H to J) Continuation of PCP degradation with time.

Acyl chain compositions of PCP-treated and nontreated cells. Table 2 shows the types of fatty acids found in membranes of *Sphingomonas* sp. strains UG30 and UG25 and *S. chlorophenolica* ATCC 39723 cultured in the presence and in the absence of 50 μg of PCP per ml. The fatty acid profile of strain ATCC 39723 is similar to that of UG25 and UG30, except for the absence of fatty acids 14:0 and 17:0 ante (methyl group at the third carbon from the end). Our results show that

there were no dramatic differences in the acyl chain contents of PCP-pretreated and control cells.

Oxygen consumption studies. Table 3 shows the results obtained when resting cells of *Sphingomonas* sp. strain UG30 were treated with different levels of PCP. With both PCP-pretreated cells and control cells, addition of 25 or 50 μg of PCP per ml enhanced the rate of oxygen consumption compared with the background (endogenous) rate. At PCP con-

FIG. 3. PCP concentrations remaining in the perfusion medium of agarose-immobilized *Sphingomonas* sp. strain UG30. See the legend to Fig. 2 and the text for details. Different symbols represent different experiments. B to E correspond to spectra in Fig. 2.

centrations greater than 75 $\mu\text{g/ml}$, however, the oxygen consumption rates declined, and at a PCP concentration of 100 $\mu\text{g/ml}$ the respiration rate was only slightly higher than the endogenous value.

Table 3 also shows the data obtained when resting cells were allowed to metabolize glutamate for 10 min before PCP was added. Initially, the rate of oxygen consumption by glutamated cells was sixfold higher than the endogenous rate observed with starved cells (see above). When higher concentrations of PCP were added to the cells, a concentration-dependent decrease in the respiration rate was observed at all of the levels tested. When the PCP concentration was 100 $\mu\text{g/ml}$, the rate of oxygen consumption was only marginally higher than the value recorded with starved cells. Since amino acid uptake in bacteria requires a transmembrane pH gradient (8), addition of progressively higher levels of PCP will gradually lower the metabolic rate.

DISCUSSION

In our batch culture studies with three PCP-degrading microorganisms, *Sphingomonas* strains UG25 and UG30 and *S. chlorophenolica* ATCC 39723, two observations were made.

FIG. 4. ^{31}P NMR spectra for *Sphingomonas* sp. strain UG30 phospholipid-deoxycholate micelles (A), PCP-pretreated *Sphingomonas* sp. strain UG30 phospholipid-deoxycholate micelles (B), *S. chlorophenolica* ATCC 39723 phospholipid-deoxycholate vesicles (C), and PCP-pretreated *S. chlorophenolica* ATCC 39723 phospholipid-deoxycholate vesicles (D). PG, CL, PE, and PC peaks were assigned by adding standard compounds. Note that the spectra in panels A and B are plotted on a different vertical scale than the spectra in panels C and D, as shown by the different signal-to-noise ratios.

TABLE 1. Chemical shift standards for phospholipids in deoxycholate vesicles

Standard	δ (ppm) ^a
α -Glycerophosphate.....	5.07
Phosphocholine	4.09
Phosphoethanolamine.....	4.58
Phosphoserine.....	4.66
Phosphatidic acid	4.10
PG	1.18
CL	1.07
PE.....	0.63
Phosphatidylserine	0.46
Phosphatidylinositol.....	0.21
PC.....	0.09

^a All chemical shifts in the ³¹P NMR spectra were determined in reference to an external MDP standard value, which was set at 18.59 ppm.

First, we observed that agarose immobilization raised the PCP degradation threshold of all three strains by 10 to 20%. Second, we observed that UG25 and UG30 cells treated for 2 h with 50 μ g of PCP per ml could subsequently degrade 30 to 50% higher levels of PCP. Strain ATCC 39723, on the other hand, had the same PCP degradation threshold regardless of whether the cells had been exposed to PCP previously.

Microorganisms encapsulated in various matrices can be protected from toxins or predators in the environment (3); thus, it was not surprising to find that our cells could degrade 20% higher PCP levels after they had been immobilized in agarose beads. Even greater levels of enhancement were observed when *Sphingomonas* sp. strain UG30 cells were entrapped in κ -carrageenan-clay beads (4). Unfortunately, the presence of paramagnetic ions in the clay amendments adversely affected the resolution of the NMR spectra. For this reason an agarose matrix, which was used successfully in previous NMR study (20), was chosen instead for this study.

The ³¹P NMR spectra obtained with agarose-immobilized UG25 and UG30 cells showed that the transmembrane pH gradient and ATP levels decreased after 120 μ g of PCP per ml

was added to the perfusion medium. The same result was observed previously when concentrated UG30 cell suspensions were treated with PCP (18). In the present study, however, we were able to monitor recovery of the transmembrane pH gradient and ATP levels. This occurred when most of the PCP in the perfusion medium had been degraded so that the concentration was around 15 μ g/ml.

When the experiment described above was repeated with PCP-pretreated UG25 and UG30 cells, the transmembrane pH gradient and ATP levels were not affected by addition of PCP to the perfusion medium. These cells were resistant to PCP deenergization, as has been reported previously for PCP-pretreated *S. chlorophenolica* ATCC 39723 (29). However, in the present study we found that in contrast to strains UG25 and UG30, PCP-pretreated ATCC 39723 cells did not have a higher PCP degradation threshold after PCP pretreatment. We were curious to understand why.

Bacteria vary in the ability to tolerate environmental toxins (16). Some strains can alter their membrane lipid compositions (5, 13, 25); others synthesize stress proteins (12) or actively excrete toxins (15). We focused on the membrane barrier as the locus for increased PCP tolerance in strains UG25 and UG30.

Acyl chain modification of the membrane phospholipids is one way in which bacteria can adapt to environmental pollutants. By increasing the concentration of saturated fatty acids or by *trans* isomerization of existing *cis* unsaturated acyl chains, membrane fluidity is decreased and compounds like PCP have a harder time penetrating the cells (5, 25). Unfortunately, commercial acyl analysis did not allow resolution of *cis* and *trans* isomers of 18:1 and 18:2 fatty acids. Thus, we were not able to determine whether *cis-trans* isomerization played an important role in increasing cellular resistance to PCP. In addition, the differences in the levels of saturated and unsaturated fatty acids between PCP-treated and untreated cells were minor and often inconsistent (Table 2). We did, however, observe major reproducible differences in membrane phospho-

TABLE 2. Fatty acid compositions of total membrane fractions

Fatty acid ^a	% in:						
	Strain ATCC 39723		Strain UG30			Strain UG25	
	PCP treated	Control	PCP treated	SD Representative	Control	PCP treated	Control
14:0	ND ^b	ND	0.6	0.17	ND	0.68	0.42
14:OH	2.79	2.68	3.83	0.49	2.09	1.91	1.86
16:1 ω 7c/15 iso 2OH	13.35	18.43	15.77	0.45	15.35	14.18	14.65
16:1 ω 5c	2.12	2.29	1.32	0.16	1.27	1.06	1.29
16:0	4.67	5.64	8.04	0.62	7.56	7.93	11.68
17:0 ante	ND	ND	0.26	0.18	0.37	ND	0.24
17:1 ω 7c	0.85	0.82	0.45	0.40	0.53	2.81	0.68
17:1 ω 6c	1.53	0.69	0.31	0.13	0.39	1.47	ND
18:2 ω 6,9c/18:0 ante	2.70	ND	0.42	0.05	1.37	3.81	0.52
18:1 ω 9c/ ω 9t/ ω 12t/ ω 7c	69.60	68.10	66.44	0.73	70.31	63.31	66.47
18:1 ω 5c	1.92	1.14	1.41	0.10	1.30	1.91	1.33
18:0	0.47	0.22	0.98	0.45	0.71	0.94	0.82

^a In the designation 18:1 ω 9c, the number before the colon indicates the total number of methylenes in the fatty acid and the number after the colon indicates the degree of unsaturation (in this case one double bond); ω 9c indicates that the double bond occurs 9 atoms from the end of the chain and has a *cis* configuration. The designation ω 12t indicates that the double bond is 12 atoms from the end and has a *trans* configuration. The designation 15 iso 2OH indicates that the fatty acid has a chain length of 15, a methyl group on the penultimate hydroxyl (carbon 14 iso position), and a hydroxyl on the 2 (α) methylene position. Ante fatty acids have a methyl group on the third carbon from the end.

^b ND, not detected.

TABLE 3. Oxygen consumption studies with *Sphingomonas* sp. strain UG30

Prepn	O ₂ consumption rate (μ mol of O ₂ /g [fresh wt] of cells/min)	
	Control cells	PCP-pretreated cells
Control	0.87	0.80
PCP (25 μ g/ml)	1.29	1.24
PCP (50 μ g/ml)	1.61	1.90
PCP (75 μ g/ml)	1.43	1.28
PCP (100 μ g/ml)	0.96	1.02
Glutamate control	6.03	5.54
Glutamate + PCP (25 μ g/ml)	5.36	5.11
Glutamate + PCP (50 μ g/ml)	5.06	4.09
Glutamate + PCP (75 μ g/ml)	3.19	2.30
Glutamate + PCP (100 μ g/ml)	1.29	1.58

lipid composition between PCP-treated and control cells with strains UG25 and UG30. Specifically, PCP-pretreated cells had a higher CL content than nontreated cells. The phospholipid profile of *S. chlorophenolica* ATCC 39723, on the other hand, was the same regardless of whether cells had been exposed to PCP.

In the bacterium *Escherichia coli*, PG is converted to CL as cultures age and when compounds such as dinitrophenol, cyanide, penicillin, and colicin K are added to the medium (7). An increase in the CL content of cell membranes has also been reported for *Pseudomonas putida* after exposure to organic solvents like toluene (25). It has been suggested that higher levels of CL increase membrane rigidity and thus present a physical barrier for the penetration of toxic compounds (28). A higher CL level in PCP-pretreated UG25 and UG30 cells would explain why these cells were subsequently able to tolerate higher PCP concentrations, whereas strain ATCC 39723, whose phospholipid composition remained unaltered, could not.

While our data showed that PCP modifies oxygen consumption (Table 3), they did not allow us to conclude whether PCP functions mainly as an uncoupler or as a respiratory inhibitor in *Sphingomonas* sp. strain UG30. Previous reports concerning the effects of PCP on cell physiology are often conflicting. PCP concentrations as low as 0.01 mM (about 3 μ g/ml) have been shown to uncouple ATP synthesis in rat liver mitochondria (32). On the other hand, PCP has been shown to associate specifically with the mitochondrial protein fraction in rat liver mitochondria (33), and studies with phenolic compounds related to PCP have demonstrated that inhibition of electron transport occurs at the cytochrome *bc*₁ complex (30). We observed that the oxygen consumption rate of resting UG30 cells depended on the PCP concentration. Similar concentration-dependent effects on respiration were observed previously when β -pinene was added to yeast mitochondria (31). Mitochondrial respiratory stimulation was observed at low concentrations of β -pinene, whereas inhibition of respiration occurred at higher concentrations, similar to the data in Table 3.

In conclusion, our results show that PCP-pretreated *Sphingomonas* sp. strains UG25 and UG30 have diminished ATP levels and reduced transmembrane pH gradients when they are exposed to PCP levels higher than 15 μ g/ml. We showed that when strains UG25 and UG30 are pretreated with 50 μ g of

PCP per ml, they can adapt to higher PCP concentrations. The adaptation process involves increasing the levels of CL in the membranes. Interestingly, the related organism *S. chlorophenolica* ATCC 39723 did not show a change in CL content when it was preexposed to PCP and subsequently did not tolerate higher PCP levels.

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REFERENCES

- Blackburn, J. W., and R. J. Harker. 1993. The impact of biochemistry, bioavailability and bioactivity on the selection of bioremediation techniques. *Trends Biotechnol.* **11**:328–333.
- Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Biophys.* **37**:911–917.
- Cassidy, M. B., H. Lee, and J. T. Trevors. 1996. Environmental applications of immobilized microbial cells. *J. Ind. Microbiol.* **16**:79–101.
- Cassidy, M. B., K. W. Shaw, H. Lee, and J. T. Trevors. 1997. Enhanced mineralization of pentachlorophenol by *k*-carrageenan-encapsulated *Pseudomonas* sp. UG30. *Appl. Microbiol. Biotechnol.* **47**:108–113.
- Clejan, S., A. A. Guffanti, L. H. Falk, and T. A. Krulwich. 1988. The protonophore resistance of *Bacillus megaterium* is correlated with elevated ratios of saturated to unsaturated fatty acids in membrane phospholipids. *Biochim. Biophys. Acta* **932**:43–51.
- Copley, S. D. 2000. The evolution of a metabolic pathway for the degradation of a toxic xenobiotic: the patchwork approach. *Trends Biochem. Soc.* **25**:261–268.
- Cronan, J. E., and P. R. Vagelos. 1972. Membrane phospholipids of *Escherichia coli*. *Biochim. Biophys. Acta* **265**:25–60.
- Decker, S. J., and D. R. Lang. 1977. Mutants of *Bacillus megaterium* resistant to uncouplers of oxidative phosphorylation. *J. Biol. Chem.* **252**:5936–5983.
- Dennis, E. A., and A. Plückthun. 1984. Phosphorus-31 NMR of phospholipids in micelles, p. 423–446. In *Phosphorus-31 NMR principles and applications*. Academic Press Inc., New York, N.Y.
- Ederer, M. M., R. L. Crawford, R. P. Herwig, and C. S. Orser. 1997. PCP degradation is mediated by closely related strains of the genus *Sphingomonas*. *Mol. Ecol.* **6**:39–49.
- Gadian, D. G. 1995. NMR and its applications to living systems, 2nd ed. Oxford University Press, Oxford, United Kingdom.
- Gage, D. J., and F. C. Neidhardt. 1993. Adaptation of *Escherichia coli* to the uncoupler of oxidative phosphorylation 2,4-dinitrophenol. *J. Bacteriol.* **175**:7105–7108.
- Heipeiper, H. J., F. J. Weber, J. Sikkema, H. Keweloh, and J. A. M. de Bont. 1994. Mechanisms of resistance of whole cells to toxic organic solvents. *Trends Biotechnol.* **12**:409–415.
- Kaback, H. R. 1971. Bacterial membranes. *Methods Enzymol.* **22**:99–120.
- Konings, W. N., B. Poolman, and A. J. M. Driessen. 1992. Can the excretion of metabolites by bacteria be manipulated? *FEMS Microbiol. Rev.* **88**:93–108.
- Krulwich, T. A., P. G. Quirk, and A. A. Guffanti. 1990. Uncoupler-resistant mutants of bacteria. *Microbiol. Rev.* **54**:52–65.
- Lee, J.-Y., and L. Xun. 1997. Purification and characterization of 2,6-dichloro-*p*-hydroquinone chlorohydrolase from *Flavobacterium* sp. strain ATCC 39723. *J. Bacteriol.* **179**:1521–1524.
- Leung, K. T., M. B. Cassidy, K. W. Shaw, H. Lee, J. T. Trevors, E. M. Lohmeier-Vogel, and H. J. Vogel. 1996. Pentachlorophenol degradation by *Pseudomonas* sp. UG25 and UG30. *World J. Microbiol. Biotechnol.* **13**:305–313.
- Leung, K. T., O. Tresse, D. Errampalli, H. Lee, and J. T. Trevors. 1997. Mineralization of *p*-nitrophenol by pentachlorophenol-degrading *Sphingomonas* sp. *FEMS Microbiol. Lett.* **155**:107–114.
- Lohmeier-Vogel, E. M., B. Hahn-Hägerdal, and H. J. Vogel. 1995. Phosphorus-31 and carbon-13 nuclear magnetic resonance studies of glucose and xylose metabolism in agarose-immobilized *Candida tropicalis*. *Appl. Environ. Microbiol.* **61**:1420–1425.
- Lohmeier-Vogel, E. M., K. Skoog, H. Vogel, and Hahn-Hägerdal. 1989. ³¹P-nuclear magnetic resonance study of the effect of azide on xylose fermentation by *Candida tropicalis*. *Appl. Environ. Microbiol.* **55**:1974–1980.
- McAllister, K. A., H. Lee, and J. T. Trevors. 1996. Microbial degradation of

- pentachlorophenol. Biodegradation 7:1–40.
23. Orser, C. S., J. Dutton, C. Lange, P. Jablonski, L. Xun, and M. Hargis. 1993. Characterization of a *Flavobacterium* glutathione S-transferase gene involved in reductive dechlorination. J. Bacteriol. 175:2640–2644.
 24. Pearce, J. M., and R. A. Komoroski. 1993. Resolution of phospholipid molecular species by ^{31}P NMR. J. Magn. Reson. Med. 29:724–731.
 25. Ramos, J. L., E. Duque, J.-J. Rodriguez-Herva, P. Godoy, A. Haidour, F. Reyes, and A. Fernandez-Barrero. 1996. Mechanisms for solvent tolerance in bacteria. J. Biol. Chem. 272:3887–3890.
 26. Robitaille, P.-M. L., P. A. Robitaille, G. G. Brown, Jr., and G. G. Brown. 1991. An analysis of the pH-dependent chemical-shift behaviour of phosphorus-containing metabolites. J. Magn. Res. 92:73–84.
 27. Saber, D. L., and R. L. Crawford. 1985. Isolation and characterization of *Flavobacterium* strains that degrade pentachlorophenol. Appl. Environ. Microbiol. 50:1512–1518.
 28. Sikkema, J., J. A. de Bont, and B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. 59:201–222.
 29. Steiert, J. G., W. J. Thoma, K. Ugurbil and R. L. Crawford. 1988. ^{31}P nuclear magnetic resonance studies of effects of some chlorophenols on *Escherichia coli* and a pentachlorophenol-degrading bacterium. J. Bacteriol. 170:4954–4957.
 30. Tokutake, N., H. Miyoshi, and T. Fujita. 1991. Electron transport inhibition by the cytochrome *bc₁* complex of rat-liver mitochondria by phenolic uncouplers. Biochim. Biophys. Acta 1057:377–383.
 31. Uribe, S., U. Ramirez, and A. Pena. 1985. Effects of β -pinene on yeast membrane functions. J. Bacteriol. 161:1195–1200.
 32. Weinbach, E. C. 1954. The effect of pentachlorophenol on oxidative phosphorylation. J. Biol. Chem. 210:545–550.
 33. Weinbach, E. C., and J. Garbus. 1965. The interaction of uncoupling phenols with mitochondria and with mitochondrial protein. J. Biol. Chem. 240:1811–1819.
 34. Xu, L., K. Resing, S. L. Lawson, P. C. Babbitt, and S. D. Copley. 1999. Evidence that *pcpA* encodes 2,6-dichlorohydroquinone dioxygenase, the ring-cleavage enzyme required for pentachlorophenol degradation in *Sphingomonas chlorophenolica* ATCC 39723. Biochemistry 38:7659–7669.
 35. Xun, L., and C. S. Orser. 1991. Purification and properties of a pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium* sp. strain ATCC 39723. J. Bacteriol. 173:4447–4453.