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Physiological properties and substrate specificity of a pentachlorophenol-degrading *Pseudomonas* species *

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Abstract

A bacterial strain capable of utilizing pentachlorophenol (PCP) as sole source of carbon and energy for growth was isolated from enrichment cultures containing 100 mg/l PCP in a mineral salts medium inoculated with contaminated soil from a lumber treatment waste site. The isolate, designated strain SR3, was identified as a species of *Pseudomonas* by virtue of its physiological and biochemical characteristics. Mineralization of PCP by *Pseudomonas* sp. strain SR3 was demonstrated by loss of detectable PCP from growth medium, stoichiometry of chloride release (5 equivalents of chloride per mole of PCP), and formation of biomass consistent with the concentration of PCP mineralized. PCP-induced cells of strain SR3 showed elevated rates of oxygen consumption in the presence of PCP, and with different chlorinated phenols, with complete degradation of 2,3,5,6-, 2,3,6-, 2,4,6-, 2,4-, and 2,6-chloro-substituted phenols. Concentrations of PCP up to 175 mg/liter supported growth of this organism, but maximal rates of PCP removal were observed at a PCP concentration of 100 mg/liter. Based on its degradative properties, *Pseudomonas* sp. strain SR3 appears to have utility in bioremediation of soil and water contaminated with PCP.

Abbreviations: DCP – dichlorophenol, TCP – trichlorophenol, TeCP – tetrachlorophenol

Introduction

Due to its widespread use as a wood-preservative, pentachlorophenol (PCP) is frequently found as a contaminant at lumber-treatment sites (Cirelli 1978; Mueller et al. 1989). Although the major use of PCP (ca. 80%) has been in the wood preserving industry, PCP and its sodium salt have also been used extensively as pesticides (Cirelli 1978). The Environmental Protection Agency (EPA) has listed PCP as a priority pollutant (Keith & Telliard 1979) and considers materials contaminated with PCP to be hazardous (US EPA 1989). The fate of PCP in the environment is of concern due to the hazards associated with its recalcitrant nature and its potential for migration and contamination of groundwater supplies.

Although PCP is recognized as an uncoupler of oxidative phosphorylation (Weinbach 1954; Imai et al. 1967), several gram-positive and gram-negative aerobic bacteria capable of degrading PCP in pure culture have been described. Gram-positive PCP-degrading organisms include strain KC–3 (Chu & Kirsch 1972), species of *Arthrobacter* (Stanlake & Finn 1982; Edgehill & Finn 1983), *Rhodococcus chlorophenolicus* (Apajalahti & Salkinoja-Salonen 1984; Apajalahti et al. 1986), and *Streptomyces rochei* (Ludmila et al. 1992). Gram-negative PCP-degrading isolates include species of *Pseudomonas* (Watanabe 1973; Suzuki

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1977; Radehaus & Schmidt 1992) and *Flavobacterium* spp. (Saber & Crawford 1985).

The initial step in PCP degradation involves its conversion to 2,3,5,6-tetrachlorohydroquinone (TeCH) as shown in strain KC–3 (Reiner et al. 1978), *Rhodococcus chlorophenolicus* (Apajalahti & Salkinoja-Salonen 1987), *Arthrobacter* sp. ATCC 33790 (Schenk et al. 1989), and *Flavobacterium* sp. ATCC 39723 (Steiert & Crawford 1986). In the latter two organisms, the PCP hydroxylase has been shown to require oxygen and NADPH for activity (Schenk et al. 1989; Xun & Orser 1991) and the purified enzyme contains 1 FAD per molecule (Xun & Orser 1991). PCP was shown to be oxidatively dechlorinated to TeCH by the purified *Flavobacterium* PCP 4-monooxygenase (Xun et al. 1992) and the gene encoding this enzyme has recently been cloned, sequenced, and expressed in *E. coli* (Orser et al. 1993).

This paper describes the isolation and PCP-related physiology of a *Pseudomonas* species designated strain SR3. The kinetics of PCP degradation and the range of chlorophenols which serve as substrates for strain SR3 are also described.

Materials and methods

Media

A basal salts minimal medium (BSM) (Hareland et al. 1975) was used for enrichment, cultivation of strain SR3, and in PCP-degradation experiments. The medium was modified for chloride-release studies by substituting ammonium sulfate (2.5 g/l) for ammonium chloride. Luria-Bertani (LB) medium (Maniatis et al. 1982) was used for non-selective strain maintainance. All media were solidified by addition of 15 g/l Bacto agar (Difco, Detroit, MI).

Enrichment and isolation of strain SR3

Pentachlorophenol-contaminated soils from former wood preserving facilities in central and northwestern Florida were used as sources of PCP-degrading microorganisms. Primary enrichment for PCP-degrading bacteria was carried out in BSM containing 40 mg/l PCP as its sodium salt. Soil was added (5% w/v) to 100 ml of medium and incubated in 500 ml Erlenmeyer flasks at 24 °C, in the dark, with rotary shaking at 200 cycles per min. Disappearance of PCP was determined spectrophotometrically by the decrease of absorbance at 320 nm as previously described (Saber & Crawford 1985). Ultraviolet spectra (200–350 nm) of aqueous supernatants were obtained after centrifugation to remove cells and suspended solids. Disappearance of PCP from the initial enrichment was first observed after 16 days in a culture containing soil from the Brown Wood Preserving Site, Live Oak, Florida. At this time, transfer of the culture (5%) was made to fresh medium. Serial subcultures were thereafter routinely transferred once disappearance of PCP was observed. After five transfers, complete PCP removal was observed within 4 days. At this point, cultures were transferred (2–5% inoculum) to medium containing 60 mg/l PCP. Within several transfers, these cultures demonstrated the ability to degrade PCP at this concentration. They were transferred in a similar fashion to BSM containing 80, 100, 113, and 125 mg/l PCP. Modified PCP-bromothymol blue indicator agar medium (Saber & Crawford 1985), containing 100 mg/l PCP and 0.5 g/l ammonium carbonate (in place of sodium nitrate), were used to isolate 3 different bacteria from cultures containing 100 mg/l PCP. One isolate, which produced an acidic reaction (a yellow zone around colonies on the blue plates), was purified on this medium and was subsequently shown to utilize PCP as sole growth substrate in liquid culture. This isolate (strain SR3) was maintained on LB agar and used in further study of its physiology and PCP-degrading properties.

Nutritional and biochemical properties

Substrate utilization by strain SR3 was determined in BSM (10 ml in 25 ml culture tubes) supplemented with various carbon substrates (0.1% (w/v)). Growth was measured turbidimetrically (540 nm) and compared to cell densities of inoculated media without carbon source addition. Additional substrate-utilization and biochemical characteristics were determined by use of Vitek AMS (Hazelwood, MO) and API Rapid NFT (Analytab Products, Plainview, NY) test systems as specified by the manufacturers. Mole percent GC analysis was determined by Tom Wahlund (Southern Illinois University, Carbondale, IL) as previously described (Wahlund et al. 1991).

Growth of strain SR3

Cells for metabolic studies were obtained using a two-step induction process: 50 mg/l PCP was added to mid-exponential phase cultures of strain SR3 grown in

BSM containing 1.0 g/l glucose and 0.2 g/l yeast extract (YE). When PCP degradation was nearly complete as shown by 320 nm absorbance, these cells were transferred (5% inoculum) to BSM containing 100 mg/l PCP, 1.0 g/l glucose, and 0.2 g/l YE. Once PCP removal ($\geq$90%) was evident, the induced cells were used as inocula or were harvested by centrifugation (8000 g, 10 min, 10 °C), washed once with BSM, and resuspended in BSM. Uninduced cells were grown in BSM containing 1.0 g/l glucose and 0.2 g/l YE. Substrates were added to BSM from autoclaved or filter-sterile stock solutions and cultures were grown in 250 ml or 500 ml Erlenmeyer flasks containing 50 or 100 ml of medium, respectively. Cultures were incubated at 24 °C, in the dark, with shaking (200 rpm) unless otherwise stated.

Chloride release studies

Chloride release during growth on PCP was measured in cultures containing 100 ml chloride-free medium (see Media) and 100 mg/l PCP. Growth was determined turbidimetrically at 540 nm and PCP concentration was determined by relating absorbance at 320 nm to a standard curve. Chloride ion concentrations were determined using a procedure adapted from the mercuric thiocyanate-dependent color reaction (procedure no. 461, Sigma Chemical Co., St. Louis, MO) to accommodate 0.1 ml samples. The modified procedure gave a linear response for chloride concentration up to 2 meq. Values shown for PCP and chloride concentrations are the means of duplicate determinations.

PCP Conversion to biomass

Conversion of PCP to biomass was determined essentially as described by Stanlake and Finn (1982). A suspension of washed SR3 cells with an optical density at 540 nm of 1.0 corresponded to 0.43 mg cell dry weight/ml. This information was used to establish a relationship between amount of PCP consumed and cell dry weight formed.

Rates of PCP degradation

Rates of PCP removal were determined in 12.5 ml BSM containing 100 mg/l PCP as a function of various dilutions of a PCP-induced cell suspension. The effect of PCP concentration on its rate of removal was similarly determined with induced cells in BSM containing from 20 to 200 mg/l PCP. Reactions were incubated at 30 °C in culture tubes (16 $\times$ 150 mm, Corning no. 9820) on an incline tube shaker (200 rpm) and 1.0 ml samples were removed for analysis of PCP. Rates were determined from measurements of PCP concentration over time.

Oxygen uptake studies

Oxygen consumption by induced and uninduced cells was measured at 30 °C with a Clark-type oxygen electrode (Gilson Medical Electronics, Middleton, WI). The electrode was calibrated with air-saturated BSM after addition of 1–2 mg of sodium dithionite. Full scale response corresponded to 225 nmol of oxygen per 1.0 ml reaction volume. The value is based on the aqueous solubility of oxygen at 30 °C (Beechey & Ribbons 1972). Washed cells were resuspended at approximately 25 mg wet wt/ml BSM. Reactions contained 1.8 ml of air-saturated BSM and 0.2 ml cell suspension and were equilibrated for 2–3 min prior to addition of substrates (0.15 mM, final concentration). Rates of oxygen consumption were corrected for endogenous respiration in the absence of substrate.

Induction and substrate specificity

The ability of induced and uninduced cells to remove different chlorophenols (CPs) was determined in BSM (6.0 ml volume) containing 0.15 mM CP with or without 100 mg/l chloramphenicol. Reactions were incubated at 30 °C in the dark with shaking. One ml samples were taken periodically over 96 h, cells were removed by centrifugation, and the supernatants analyzed for substrate removal after filtration (0.2 um Acrodisc filters, Gelman) by UV and HPLC. Results are corrected for losses observed in uninoculated controls. High-performance liquid chromatography (HPLC) was performed on a Hewlett-Packard model 1090M equipped with a diode array detector and a Hypersil-ODS reverse phase (100 $\times$ 2.1 mm) column (Hewlett-Packard Co., Palo Alto, CA). The mobile phase was 50 mM KH_2PO_4, pH 3.5 (A) and acetonitrile (B) at a flow rate of 0.5 ml/min. The initial concentration of B was 10% and increased at 3% per min to 55% in 15 min. The injection volume was 10.0 μl. The detector wavelength was 207 nm and peak areas, retention times, and ultraviolet absorption spectra (200–350 nm) were compared with those of authentic standards.

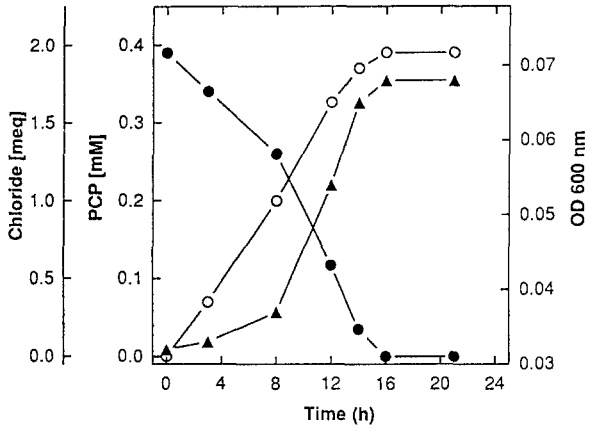

Fig. 1. Release of inorganic chloride during growth of *Pseudomonas* sp. strain SR3 on 100 mg/l PCP. Symbols: •, PCP concentration; o, chloride concentration; ▲, culture turbidity (OD 600 nm). Refer to text for details.

Chemicals and reagents

Pentachlorophenol was from JT Baker Chemical Co. (Phillipsburg, NJ). All chlorophenols were of the highest purity available from Aldrich Chemical Co. (Milwaukee, WI). Chloramphenicol and the reagent for chloride determination were from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest purity commercially available.

Results and discussion

Serial enrichments led to the isolation of a PCP-degrading bacterium from PCP-contaminated soil. A PCP-degrading population was initially established in media containing 40 mg/l PCP as sole source of carbon and energy. Transfers were made to increasing PCP concentrations as the PCP was more rapidly degraded. Due to the low biomass resulting from growth on PCP as sole carbon source, PCP degradation was assessed by measuring the loss of absorbance at 320 nm rather than by an increase in cell density. PCP-bromothymol blue indicator plates were used to isolate a gram-negative PCP-degrading bacterium characterized as a species of *Pseudomonas* and designated strain SR3. Carbon sources which supported growth of strain SR3 include acetate, L-arabinose, L-aspartate, cellobiose, D-galactose, D-glucose, L-glutamate, L-malate, succinate, and PCP. Among the compounds not utilized are adipate, L-alanine, L-arginine, caprate, citrate, D-gluconate, L-glutamine, lactate, maltose, D-mannitol, D-mannose, N-acetyl-D-glucosamine, phenylacetate, and L-serine. Strain SR3 was positive for oxidase, catalase, β-galactosidase, esculin hydrolysis, and negative for glucose fermentation, L-arginine dihydrolase, urease, gelatinase, and nitrate reduction. Cells of strain SR3 were motile, rod shaped (0.5 × 1.3 μm), and grew slowly to produce opaque, off-white colored colonies on LB and chemically defined media. Thermal denaturation of nucleic acids revealed a mole percent GC content of 64.2 for strain SR3, consistent with the assignment of this organism to the genus *Pseudomonas*. Although the carbon sources utilized by strain SR3 correlate closely with those utilized by the type strain of *P. vesicularis*, ATTC 11426 (Ballard et al. 1968; Palleroni 1984) (an assignment supported by both Vitek AMS and API-NFT tests), analysis of the fatty acid methyl ester profile indicated that strain SR3 was more closely related to members of the *Pseudomonas* rRNA subgroup III (Palleroni 1984) (M. Sasser, pers. comm.).

Complete dehalogenation of PCP by strain SR3 was demonstrated by release of chloride corresponding to the disappearance of PCP. A stoichiometry of five equivalents of chloride released for every mole of PCP consumed is shown by the formation of 1.95 meq chloride from 0.39 mM PCP (Fig. 1). Despite the low cell densities achieved, turbidimetric measurements provided the basis for a growth curve. Cell yields resulting from growth with various limiting concentrations of PCP showed a linear relationship between cell dry weight and PCP concentration (data not shown). Growth at the expense of PCP occurred at concentrations up to 175 mg/l. A yield of 28.4 g cell dry wt/mole PCP represents a 10.7% conversion of PCP to biomass. The cell yield of 0.11 g/g PCP for strain SR3 during growth exclusively on PCP is similar to the yield of 0.15 g/g PCP reported for *Arthrobacter* sp. strain NC (Stanlake & Finn 1982).

PCP-degradation measured in reactions containing 100 mg/l PCP and varying cell densities showed that the rate of PCP-degradation was a direct function of cell concentration (Fig. 2). The highest cell density employed (0.45 mg cell dry weight/ml) showed complete PCP degradation within 2 hours while reactions containing lower cell densities gave correspondingly reduced rates of PCP-degradation. The linear relationship between PCP degradation rate and cell density (inset of Fig. 2) allows calculation of a degradation rate of 540 nmol PCP · mg dry $wt^{-1} \cdot h^{-1}$.

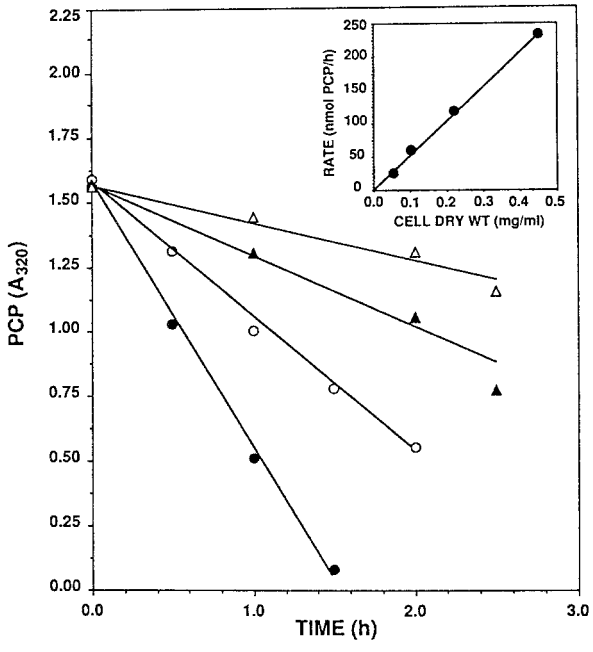

Fig. 2. Degradation of 100 mg/l PCP by PCP-induced cells of strain SR3 at 0.45 (•), 0.22 (○), 0.10 (▲), and 0.05 (△) mg cell dry wt/ml. Inset shows the relationship between the rate of PCP degradation and cell density.

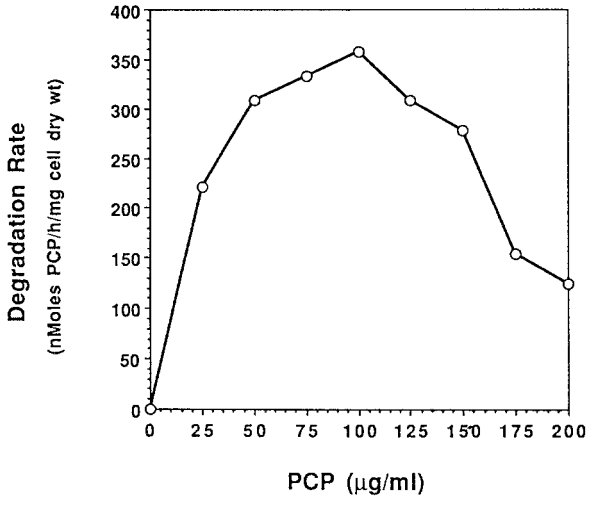

Fig. 3. Rate of PCP degradation by strain SR3 as a function of PCP concentration. Reactions containing PCP-induced cells (A_{540} = 0.84; 0.36 mg dry wt/ml) were sampled at 15 minute intervals and PCP-degradation rates were determined from the initial linear portions of the plotted disappearance curves (at concentrations of 125 mg/l and greater, a brief lag preceded the measured linear degradation rate).

When rates of PCP degradation were measured as a function of its concentration, induced cells showed maximal rates of PCP removal at 100 mg/l PCP (357 nmole PCP · mg cell dry wt^{-1} · h^{-1}) (Fig. 3). Rates within 10% of this maximum were observed over a concentration range of 75 to 150 mg/l PCP, but decreased considerably at PCP concentrations below 50 mg/l and greater than 150 mg/l. At 200 mg/l PCP, the rate of PCP removal was about 30% of the maximum. Differences in the extent of induction of strain SR3 may be responsible for differences in the maximal rates of degradation of PCP noted in Figs 2 and 3.

Elevated rates of oxygen consumption in the presence of PCP were observed only with PCP-induced cells (Table 1). Uninduced cells failed to oxidize PCP. PCP-induced cells oxidized 2,3,6-TCP and 2,4,6-TCP at rates similar to that of PCP, with lower but significant rates of oxidation observed with 2,3-DCP, 2,4-DCP, 2,6-DCP and 2,3,4,6-TeCP. 2,3,4-TCP and 3,4-DCP also were oxidized at rates significantly higher than those found with uninduced cells. Oxygen uptake with monochlorophenols was low with both induced and uninduced cells. Certain chlorophenols with a 3,5-substitution pattern, such as 2,3,4,5-TeCP, 2,3,5-TCP and 3,5-DCP, were not oxidized and 3,4,5-TCP inhibited endogenous respiration. An inhibitory effect on respiration of uninduced cells was also seen with 3,4,5-TCP and with 2,3,5,6-TeCP. Uninduced cells oxidized other chlorophenols at low rates or not at all.

To determine if oxygen uptake rate was a reliable indication of substrate removal, concentrations of chlorophenols were measured after incubation with induced and uninduced cells (Table 2). PCP and 2,4,6-TCP were completely removed by induced cells within 5 hours in the absence or presence of chloramphenicol. In the presence of chloramphenicol, uninduced cells effected no change in any chlorophenol concentration, but without chloramphenicol, an inducible response resulted in the complete loss of PCP. Several other chlorophenols were also capable of inducing their own degradation; in the absence of chloramphenicol, 2,4-DCP, 2,6-DCP, 2,3,6-TCP, and 2,4,6-TCP were completely removed. This induction may explain how chlorophenols such as 2,4-DCP, 2,6-DCP, 2,3,6-TCP, and 2,3,5,6-TeCP, which were only partially degraded in the presence of chloramphenicol, were completely degraded by PCP-induced cells in the absence of chloramphenicol (Table 2). PCP-induced cells of *Pseudomonas* sp. strain SR3 completely removed PCP, 2,3,5,6-TeCP, 2,3,6-TCP, 2,4,6-TCP, 2,4-DCP and 2,6-

Table 1. Oxygen consumption in the presence of chlorophenols by *Pseudomonas* sp. strain SR3.

Substrate (0.15 mM)	Rates of oxygen consumption[1]	
	PCP-induced cells	Uninduced cells
PCP	23	0
2,3,4,5-TeCP	0	I[3]
2,3,4,6-TeCP	16	0
2,3,4-TCP	11	5
2,3,5-TCP	0	0
2,3,6-TCP	29	10
2,4,5-TCP	ND[2]	2
2,4,6-TCP	26	2
3,4,5-TCP	I[3]	I[3]
2,3-DCP	13	6
2,4-DCP	15	6
2,5-DCP	2	4
2,6-DCP	17	2
3,4-DCP	11	6
3,5-DCP	0	ND[2]
2-CP	5	0
3-CP	2	2
4-CP	2	0

[1]Results are expressed as nanomoles · minute^{-1} · milligram cell dry weight^{-1} and are corrected for endogenous activity in the absence of substrate (4–6 nmol O_2 · min^{-1} · mg dry wt^{-1}). Values are the means of duplicate determinations.

[2]ND = not determined.

[3]I = endogenous respiration was inhibited by the substrate at concentration tested.

Table 2. Removal of chlorophenols by *Pseudomonas* sp. strain SR3 cells

Substrate (0.15 mM)	Percentage of compound removed[1]			
	PCP-induced cells[2]		Uninduced cells[3]	
	- Cm	+ Cm	- Cm	+Cm
PCP	100 (5 h)	100 (5 h)	100	0
2,3,5,6-TeCP	100	6	5	0
2,3,4-TCP	0	ND[4]	ND	ND
2,3,6-TCP	100 (5 h)	41	100	0
2,4,6-TCP	100 (5 h)	100 (5 h)	100 (48 h)	0
2,3-DCP	0	0	0	0
2,4-DCP	100 (5 h)	26	100 (48 h)	0
2,5-DCP	0	0	0	0
2,6-DCP	100 (5 h)	28	100	0
3,4-DCP	0	ND	ND	ND

[1]Determined by UV/HPLC analysis of reactions with, and without, 100 mg/l chloramphenicol (Cm) and corrected for abiotic losses.

[2]Reactions contained 0.43 mg cell dry wt/ml of PCP-induced cells and were incubated for 72 h (or 5 h where shown).

[3]Reactions contained 0.72 mg cell dry wt/ml of uninduced cells and were incubated for 96h (or 48h where shown).

[4]ND = not determined.

DCP. Each of these, with the exception of 2,3,5,6-TeCP, appears capable of inducing its own degradation (see uninduced cell columns, Table 2).

Generally, chlorophenols having 2,4- or 2,6-chloro-substitution patterns were better substrates than 3,5-substituted chlorophenols. Similar patterns of chlorophenol degradation in which preferred degradation occurs for 2,6-substituted phenols have been observed with cells of strain KC–3 (Chu & Kirsch 1973) and *Flavobacterium* sp. ATCC 39723 (Steiert et al. 1987), and with cell extracts from *Arthrobacter* sp. ATCC 33790 (Schenk et al. 1989). In addition, the gene encoding the PCP 4-monooxygenase from *Flavobacterium* sp. ATCC 39723 hybridizes with genomic DNA from both *Pseudomonas* sp. strain SR3 and *Arthrobacter* sp. ATCC 33790 (Orser et al. 1993). This finding supports the similar patterns of chlorophenol-degrading substrate specificity of these bacteria. The observation that 2,4-DCP is degraded by strain SR3, but not by *Flavobacterium* sp. ATCC 39723 cells (Steiert et al. 1987) or *Arthrobacter* sp. ATCC 33790 cell extracts (Schenk et al. 1989), may indicate differences in substrate specificity, toxicity, or transport among these strains.

Three compounds, 2,3,4-TCP, 2,3-DCP and 3,4-DCP, were not removed by PCP-induced cells, even though rates of oxygen consumption were elevated in their presence (Table 2). Thus oxygen uptake was not a reliable indication of substrate utilization. In cases where chlorophenols previously shown to stimulate rates of respiration were not removed, the compounds may have acted as uncouplers of oxidative phosphorylation as suggested previously (Steiert et al. 1987). Alternatively, these chlorophenols may stimulate oxygen consumption without themselves being acted upon (Ribbons et al. 1971).

Its ability to degrade PCP and its broad substrate specificity with respect to chlorophenols make *Pseudomonas* sp. strain SR3 potentially useful for bioremediation of PCP-contaminated soil and groundwater. Induced cells of strain SR3 actively degrade PCP in the presence of high molecular weight polycyclic aromatic hydrocarbons from creosote (Resnick & Chapman,

unpublished data). The strain has been used to remove PCP from creosote- and PCP-contaminated groundwater (Mueller et al. 1993), and to effectively reduce toxicity of PCP-contaminated water (Middaugh et al. 1993).

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