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# Substrate-dependent autoaggregation of *Pseudomonas putida* CP1 during the degradation of mono-chlorophenols and phenol

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**A bacterium, CP1, identified as *Pseudomonas putida* strain, was investigated for its ability to grow on and degrade mono-chlorophenols and phenols as sole carbon sources in aerobic shaking batch culture. The organism degraded up to 1.56 mM 2- and 3-chlorophenol, 2.34 mM 4-chlorophenol and 8.5 mM phenol using an *ortho*-cleavage pathway. *P. putida* CP1, acclimated to degrade 2-chlorophenol, was capable of 3-chlorocatechol degradation, while *P. putida*, acclimated to 4-chlorophenol degradation, degraded 4-chlorocatechol. Growth of *P. putida* CP1 on higher concentrations of the mono-chlorophenols, ≥1.56 mM 4-chlorophenol and ≥0.78 mM 2- and 3-chlorophenol, resulted in decreases in cell biomass despite metabolism of the substrates, and the formation of large aggregates of cells in the culture medium. Increases in cell biomass with no clumping of the cells resulted from growth of *P. putida* CP1 on phenol or on lower concentrations of mono-chlorophenol. Bacterial adherence to hydrocarbons (BATH) assays showed cells grown on the higher concentrations of mono-chlorophenol to be more hydrophobic than those grown on phenol and lower concentrations of mono-chlorophenol. The results suggested that increased hydrophobicity and autoaggregation of *P. putida* CP1 were a response to toxicity of the added substrates.**
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**Keywords:** mono-chlorophenol; phenol; *Pseudomonas putida* CP1; *ortho*-cleavage pathway; aggregation; hydrophobicity; toxicity

## Introduction

Phenol and its mono-chlorinated derivatives are pollutants of environmental concern due to their use in a wide variety of agricultural and industrial processes [19]. Phenol is found in refinery and chemical plant wastewaters [28], while two of the mono-chlorophenol isomers find uses in industry. 2-Chlorophenol is produced as an intermediate in the production of phenols, phenol resins and dyes and is used as a bactericide and fungicide, while 4-chlorophenol is an intermediate in the production of 2,4-dichlorophenol, trichlorophenol and tetrachlorophenol and is also used as a solvent for the mineral oil industry [35]. The acute toxicity of chlorinated phenols has led them to become priority pollutants for both the World Health Organisation and the US Environmental Protection Agency [10]. The influx of such phenolic compounds into waste treatment systems can inhibit microbial activity and may result in deterioration of treatment performance and, in extreme cases, complete breakdown of wastewater treatment [30].

The relatively high water solubility of phenolics, coupled with their recalcitrance, has led to their contamination of many environments. While the aerobic degradation of phenol has been widely reported, complete removal of all three mono-chlorophenols by a single degradative system has rarely been demonstrated [15,22,36]. The initial step in the aerobic degradation of phenolic compounds is their transformation to catechols by the enzyme phenol hydroxylase. Following their transformation to catechols, cleavage of the aromatic ring occurs using either the *ortho*- or the *meta*-cleavage pathway. Phenol, which is initially transformed to catechol, may be completely degraded using either pathway, although the *meta*-pathway is more common [16]. Mono-chlorophenols are initially transformed to chlorocatechols following which ring cleavage occurs. *Meta*-cleavage of chlorocatechols generally results in the production of coloured, dead-end metabolites resulting in incomplete degradation [3]. The complete degradation of 3- and 4-chlorocatechol *via* a *meta*-cleavage pathway has been demonstrated, but is extremely rare [13,18,23,33]. Consequently, the modified *ortho*-cleavage pathway is usually necessary for complete degradation of mono-chlorophenols [20].

The ability of microbes to aggregate is one of great importance in the bioremediation of toxic chemicals, particularly in the activated sludge process. Microbes capable of flocculation have distinct advantages over nonflocculating organisms as they may remain within sludge units for longer periods of time through biomass recycling, while the flocs form a protection against predatory protozoa, which feed mainly on free-swimming microbes [34]. A number of theories have been proposed for the autoaggregation of microorganisms, including the presence of substrate gradients, slow growth of the organism, physical/chemical stress and predation, although aggregation is not yet fully understood [6]. In this study, a naturally isolated strain was identified and examined for its ability to grow on and degrade phenol and all three mono-chlorophenols. The isolate, identified as a strain of *Pseudomonas putida*, demonstrated substrate- and concentration-dependent autoaggregation, which was also investigated.

## Materials and methods

### Chemical

2-, 3-and 4-Chlorophenol used in degradation studies, as well as minimal medium components, were obtained from Sigma-Aldrich Chemical (Dorset, UK). 3- and 4-Chlorocatechol were obtained from Helix Biotech (Richmond, BC, Canada).

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### Organism

The organism, CP1, obtained from Dr. Fabio Fava (University of Bologna, Italy), was identified in our laboratory using an AP1 20NE system (BioMérieux, Marcy-1'Etoile, France) and BIOLOG GN2 microplates (BIOLOG, Hayward, CA, USA). The identification was confirmed by analysis of some biochemical characteristics assayed according to Cowan and Steel's Manual for the Identification of Medical Bacteria [2]. CP1 was separately maintained on 2-chlorophenol (1.56 mM), 3-chlorophenol (0.78 mM), 4-chlorophenol (1.56 mM) or phenol (1.56 mM) minimal medium agar plates.

### Media and culture conditions

Biodegradation experiments were performed in 250-ml conical flasks containing 100 ml of minimal medium. The medium used in the degradation studies contained (g/l) $K_2HPO_4$, 4.36; $NaH_2PO_4$, 3.45; $(NH_4)_2SO_4$, 1.26; $MgSO_4 \cdot 6H_2O$, 0.912; and trace salts solution, 1 ml/l. The trace salts solution contained (g/100 ml): $CaCl_2 \cdot 2H_2O$, 4.77; $FeSO_4 \cdot 7H_2O$, 0.37; $CoCl_2 \cdot 6H_2O$, 0.37; $MnCl_2$, 0.10; and $Na_2MoO_4 \cdot 2H_2O$, 0.02. The pH of the medium was adjusted to 7.0 and the medium was autoclaved at 121°C for 15 min. Phenol, chlorophenols or chlorocathecols were added to flasks following sterilisation of the medium.

*P. putida* CP1 was grown overnight in nutrient broth, washed twice with 0.01 M sodium phosphate buffer, pH 7.0, and resuspended to give an optical density (OD) at 660 nm of 0.7. The flasks were inoculated with a 5% vol/vol inoculum, which corresponded to approximately $4 \times 10^7$ cells/ml, and were incubated at 150 rpm at 30°C. Samples were removed aseptically at regular intervals and analysed for chlorophenol/phenol/chlorocatechol concentrations and bacterial adherence. Uninoculated control flasks were incubated in parallel. No drop in phenol, or 3- and 4-chlorophenol concentrations was found in the uninoculated control flasks over the course of the biodegradation experiments, while the average loss in 2-chlorophenol concentrations due to volatilisation was 4 $\mu$M/h. All values were corrected to account for any volatilisation losses.

### Analytical methods

Measurement of chlorophenols was carried out using a 4-aminoantipyrene colorimetric method based on the procedure detailed in Standard Methods for the Examination of Water and Wastewater [9].

Chlorocatechol concentrations were quantified by the method of Arnow [1].

Chloride release was followed using an Orion chloride-specific electrode (model 9417). Chloride concentrations were calculated with reference to a standard curve constructed with NaCl standards. Samples and standards were diluted with 2% ionic strength adjusting buffer (5 M $NaNO_3$).

OD measurements were carried out using an ATI Unican spectrophotometer.

Substrate-dependent *P. putida* CP1 cell morphologies were photographed using a Hewlett Packard Photosmart digital camera (model C200).

Dry weight measurements were determined by filtering a specific volume of suspended culture through preweighed 0.45-$\mu$m pore size filters (Pall, Ann Arbor, MI), drying the cells at 105°C for 2 h and reweighing them.

Floc areas were determined by placing a sample in a Petri dish, capturing an image using a Hewlett Packard flat bed scanner against a black background and calculating floc areas using an Optimas 6.5 image analysis computer package.

### Bacterial adherence to hydrocarbons (BATH)

The hydrophobicity of *P. putida* CP1 during growth on phenol/chlorophenols was determined using the method of Rosenberg *et al* [26]. Cells were removed by centrifugation (3200×*g*, 10 min) and washed twice with 5 mg/l sodium tripolyphosphate buffer to remove any interfering solutes. The cells were resuspended with sodium tripolyphosphate buffer to give a final OD at 400 nm of approximately 0.6. Four milliliters of the washed cells was added to acid-washed test tubes and overlaid with 1 ml of *n*-octane. The resultant aqueous/organic mixtures were mixed with a vortex mixer for 1 min and the mixtures were allowed to settle for 15 min. The aqueous phase was then carefully removed using a Pasteur pipette and the OD at 400 nm was read. Hydrophobicity expressed as adherence to *n*-octane was calculated using the formula: BATH=100×[(OD 400 nm of washed cells)−(OD 400 nm of aqueous layer following extraction with *n*-octane/OD 400 nm of washed cells)] [31].

### Enzyme assays

Cells grown on phenol/mono-chlorophenols were harvested by centrifugation (3200×*g*, 10 min) and washed twice with 0.33 M Tris–HCl buffer (pH 7.6). The cells were broken by sonication and centrifuged at 25,000×*g*, 0–4°C for 20 min. The cell-free extract was kept on ice and assayed as soon as possible for catechol dioxygenase activity using the method of Feist and Hegeman [16].

Catechol 1,2-dioxygenase activity was measured by following the formation of *cis*,*cis*-muconic acid, the *ortho*-cleavage product of catechol. The following reagents were added to a quartz cuvette: 2 ml of 50 mM Tris–HCl buffer (pH 8.0); 0.7 ml of distilled water; 0.1 ml of 100 mM 2-mercaptoethanol and 0.1 ml of cell-free extract. The contents of the cuvette were mixed by inversion and 0.1 ml of catechol (1 mM) was then added and the contents mixed again. *Cis*,*cis*-muconic acid formation was followed by an increase in the absorbance at 260 nm over a period of 5 min.

Chlorocatechol 1,2-dioxygenase activity was measured by following the formation of 2-chloromuconic acid, the *ortho*-cleavage product of 3-chlorocatechol. The procedure used was as for catechol 1,2-dioxygenase activity, with 3-chlorocatechol (1 mM) being used in place of catechol (1 mM).

Catechol 2,3-dioxygenase activity was measured by following the formation of 2-hydroxymuconic semialdehyde, the *meta*-cleavage product of catechol. The following reagents were added to plastic cuvette: 2 ml of 50 mM Tris–HCl buffer (pH 7.5), 0.6 ml of distilled water and 0.2 ml of cell-free extract. The contents were mixed by inversion and 0.2 ml of catechol (100 mM) was added and mixed with the contents. 2-Hydroxymuconic semialdehyde production was followed by an increase in absorbance at 375 nm over a period of 5 min.

Activities were calculated using the following extinction coefficients for each reaction product: catechol at 260 nm=16,800 l/mol/cm, 3-chlorocatechol at 260 nm=17,100 l/mol/cm [27] and catechol at 375 nm=36,000 l/mol/cm [33]. One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 $\mu$mol of product per minute. Specific activities were expressed as units per milligram of protein. The

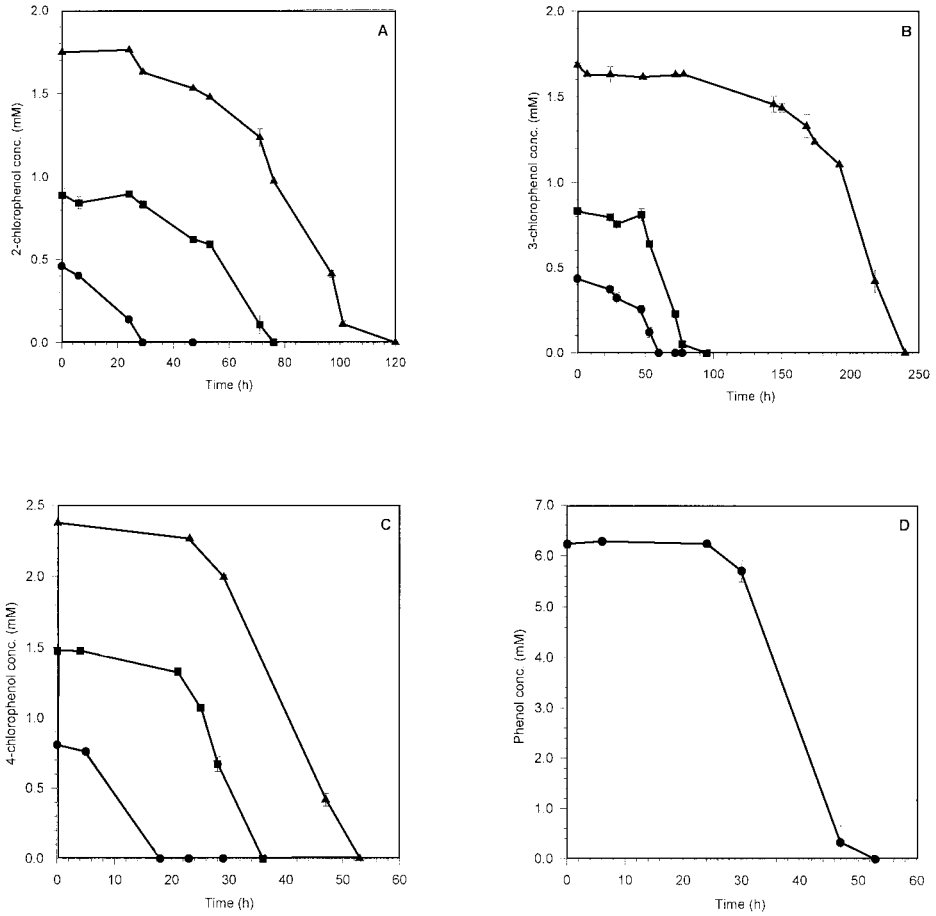


**Figure 1** Changes in chlorophenol concentrations during the degradation of (A) 2-chlorophenol; (B) 3-chlorophenol: (●) 0.39 mM, (■) 0.78 mM and (▲) 1.56 mM; (C) 4-chlorophenol: (●) 0.78 mM, (■) 1.56 mM and (▲) 2.34 mM; and (D) phenol: (●) 8.5 mM, by *P. putida* CP1.

protein concentrations in cell-free extracts were determined by the method of Lowry *et al* [21].

## Results

### *Identification of CP1 strain*

The chlorophenol-degrading isolate was a Gram-negative, non-sporulating, oxidative, motile rod. It was capable of growth on cetrimide agar, fluoresced on *Pseudomonas* F agar and was both oxidase- and catalase-positive. The strain gave positive results for arginine hydrolysis and malonate utilisation and negative results for starch hydrolysis and Tween 80 hydrolysis. On the basis of these biochemical characteristics and identification using API 20NE tests and by the BIOLOG identification system, the isolate was identified as a *P. putida* strain and was given the distinguishing code CP1.

### *Degradation of mono-chlorophenols and phenol*

Degradation of the mono-chlorophenol isomers and phenol by *P. putida* CP1 is shown in Figure 1. *P. putida* CP1 degraded up to

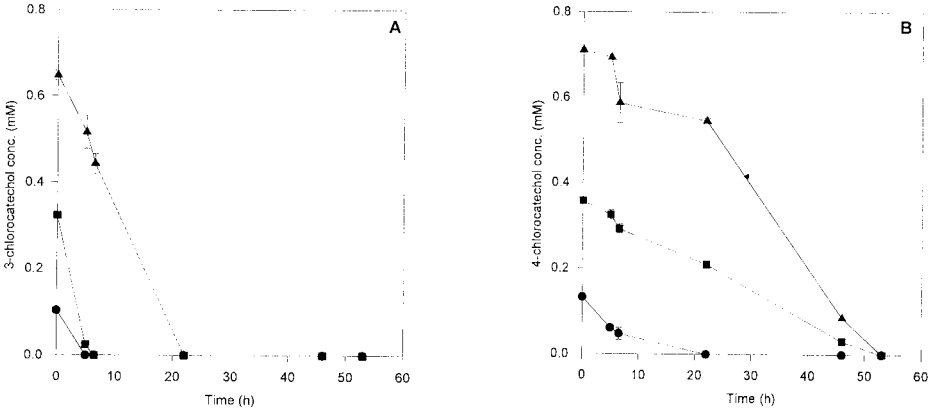


**Figure 2** Degradation of (A) 3-chlorocatechol and (B) 4-chlorocatechol: (●) 0.13mM, (■) 0.35 mM and (▲) 0.69 mM, by *P. putida* CP1.

**Table 1** Maximum ring cleavage activities during the degradation of mono-chlorophenols and phenol by *P. putida* CP1

| Enzyme assayed and assay substrate | Specific activity (U/mg) following growth on | | | |
|---|---|---|---|---|
| | 2-cp | 3-cp | 4-cp | Phenol |
| *Catechol 1,2-dioxygenase* | | | | |
| Catechol | 5.92 | 5.55 | 6.63 | 447.73 |
| 3-Chlorocatechol | 4.06 | 3.35 | 5.55 | 8.17 |
| *Catechol 2,3-dioxygenase* | | | | |
| Catechol | <0.001 | <0.001 | <0.001 | <0.001 |

1.56 mM 2-chlorophenol within approximately 104 h (Figure 1A), but it was unable to degrade 2.34 mM 2-chlorophenol. 3-Chlorophenol was less readily degraded than 2-chlorophenol, with up to 1.56 mM 3-chlorophenol being degraded within 240 h (Figure 1B). A concentration of 2.34 mM 3-chlorophenol was not degraded by *P. putida* CP1, but it completely degraded up to 2.34 mM 4-chlorophenol within 53 h (Figure 1C). A concentration of 3.12 mM 4-chlorophenol was not degraded. At a single concentration of 0.78 mM mono-chlorophenol, *P. putida* CP1 degraded 2-chlorophenol within 72 h, 3-chlorophenol within 80 h and 4-chlorophenol within 30 h, indicating the ability of *P. putida* CP1 to degrade mono-chlorophenols as in the order 4-chlorophenol>2-chlorophenol>3-chlorophenol. A similar trend was also observed at the single concentration of 1.56 mM. For each of the mono-chlorophenols, degradation by *P. putida* CP1 was accompanied by concurrent, stoichiometric releases of chloride (data not shown). Phenol degradation was greater than mono-chlorophenol degradation with up to 8.5 mM phenol being degraded by *P. putida* CP1 within 53 h (Figure 1D). During degradation of phenol and the mono-chlorophenols, no colours due to accumulation of polymerised catechol intermediates were observed in the culture medium.

## Degradation of 3- and 4-chlorocatechol

Although no chlorocatechols were detected during the degradation of 2-, 3- and 4-chlorophenol by *P. putida* CP1, 3-chlorocatechol is a known intermediate of the degradation of 2- and 3-chlorophenol, while 4-chlorocatechol is produced during the degradation of 4-chlorophenol [17]. Therefore, it was of interest to study the degradation of 3-/4-chlorocatechol. Cells capable of the degradation of 2-chlorophenol were tested for their ability to degrade 3-chlorocatechol, while cells capable of the degradation of 4-chlorophenol were used to study 4-chlorocatechol degradation. *P. putida* CP1 was shown to be capable of degrading 0.13 and 0.35 mM 3-chlorocatechol within 5 h, and 0.69 mM 3-chlorocatechol within 20 h (Figure 2A). During the degradation of 0.69 mM 3-chlorocatechol, a faint brown colour developed in the culture medium, indicating oxidative polymerisation of the

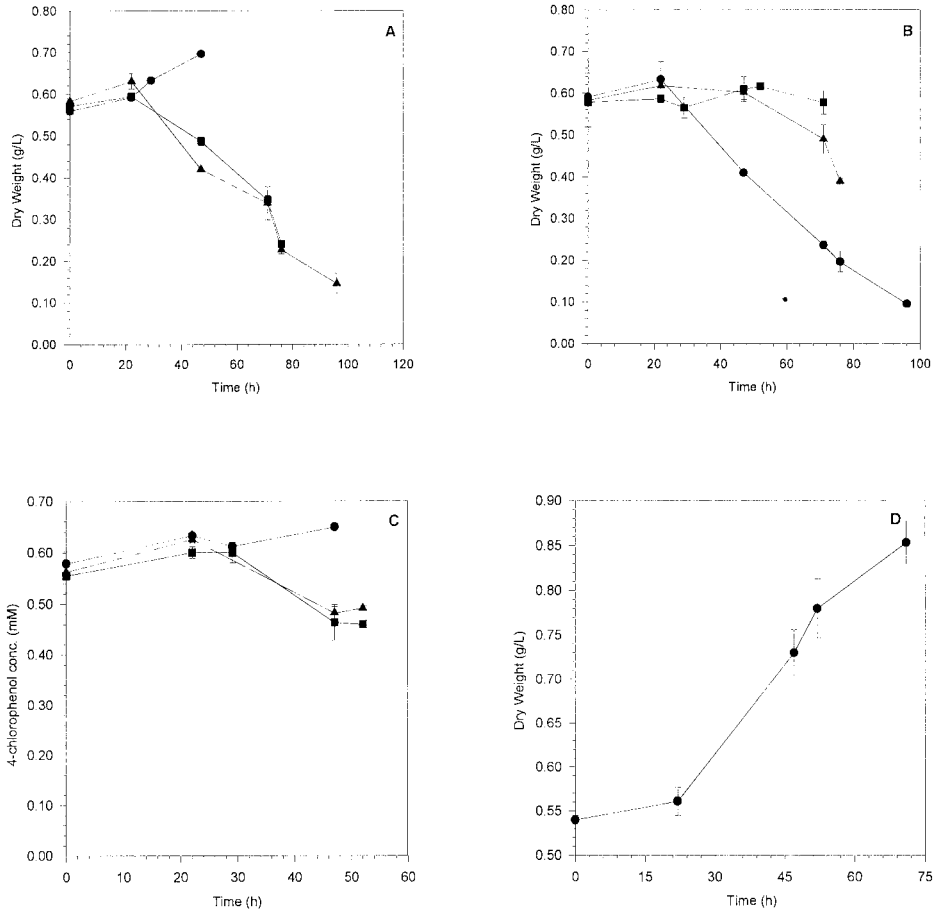


**Figure 3** Changes in dry weight during the degradation of (A) 2-chlorophenol; (B) 3-chlorophenol: (●) 0.39 mM, (■) 0.78 mM and (▲) 1.56 mM; (C) 4-chlorophenol: (●) 0.78 mM, (■) 1.56 mM and (▲) 2.34 mM; (D) phenol: (●) 8.5 mM, by *P. putida* CP1.

chlorocatechol [3]. Uninoculated control flasks containing 0.69 mM 3-chlorocatechol also developed a brown coloration in the culture medium. Due to polymerisation of the chlorocatechol, only 82% total chloride release was observed during the degradation of 0.69 mm 3-chlorocatechol, while degradation of 0.13 and 0.35 mM 3-chlorocatechol resulted in stoichiometric releases of chloride.

Degradation of 4-chlorocatechol was slower than that observed for 3-chlorocatechol. *P. putida* CP1 was capable of degrading 0.13, 0.35 and 0.69 mM 4-chlorocatechol within 10, 46 and 53 h, respectively (Figure 2B). Polymerisation was also observed in flasks containing 0.69 mM 4-chlorocatechol, resulting in a slight brown coloration of the medium. Similarly, uninoculated controls containing 0.69 mM 4-chlorocatechol developed a brown coloration of the culture medium. Degradation of 0.13 and 0.35 mM 4-chlorocatechol by *P. putida* CP1 resulted in stoichiometric releases of chloride, while degradation of 0.69 mM 4-chlorocatechol resulted in 90% total chloride release.

### *Analysis of the ring cleavage activities during the degradation of mono-chlorophenols and phenol*

The absence of colour production in the culture media due to the accumulation of dead-end metabolites and the stoichiometric releases of chloride indicated that degradation of the mono-chlorophenols was complete, suggesting that phenolic degradation occurred using the *ortho*-cleavage pathway. Analysis of the ring cleavage enzymes carried out following growth on 1.56 mM 2-/4-chlorophenol, 0.78 mM 3-chlorophenol and 8.5 mM phenol confirmed this (Table 1). *Ortho*-cleavage activities were detected during growth, on phenol and the mono-chlorophenols. *P. putida* CP1 grown on phenol showed predominantly *ortho*-cleavage activity towards catechol, while *P. putida* CP1 grown on the mono-chlorophenols displayed similar *ortho*-cleavage activities towards catechol and 3-chlorocatechol. No *meta*-cleavage activity was detected during the degradation of either phenol or the mono-chlorophenols.

### *Growth of P. putida CP1 on mono-chlorophenols and phenol*

Incubation of *P. putida* CP1 on lower concentrations of mono-chlorophenols (0.39 mM 2-and 3-chlorophenol and 0.78 mM 4-chlorophenol) and phenol resulted in increases in cell biomass corresponding to metabolism of the substrates (Figure 3A–D). However, when mono-chlorophenol concentrations were increased ($\geq$0.78 mM 2- and 3-chlorophenol and $\geq$1.56 mM 4-chlorophenol), incubation of *P. putida* CP1 with the mono-chlorophenols resulted in a decrease in cell biomass despite metabolism of the mono-chlorophenols (Figure 3A–C). Cell biomass decreases during the degradation of 2- and 3-chlorophenol were greater than those observed during 4-chlorophenol degradation. The morphology of *P. putida* CP1 in liquid culture during the degradation of phenolics varied with the growth substrate and its concentration. During growth on the mono-chlorophenols at concentrations of 0.78 mM and above for 2- and 3-chlorophenol and at concentrations at 1.56 mM and above for 4-chlorophenol, large clumps of cells were formed in the culture medium (Figure 4A–C). Aggregation occurred during the lag period of chlorophenol degradation and degradation did not occur until clumps had been formed and corresponded to decreases in cell biomass. Growth on 4-chlorophenol resulted in visible flocs throughout the culture medium with areas up to 4 $mm^2$, while growth on 2- and 3-chlorophenol resulted in larger, less numerous flocs with maximum areas up to 14 $mm^2$ during growth on 2-chlorophenol and 16 $mm^2$ on 3-chlorophenol. No clumps were formed during growth on the lower concentrations of 0.39 mM 2- and 3-chlorophenol and 0.78 mM 4-chlorophenol. During growth on phenol at concentrations up to 8.5 mM, no clump formation was noted (Figure 4D). Similarly, no clumping was observed during the degradation of

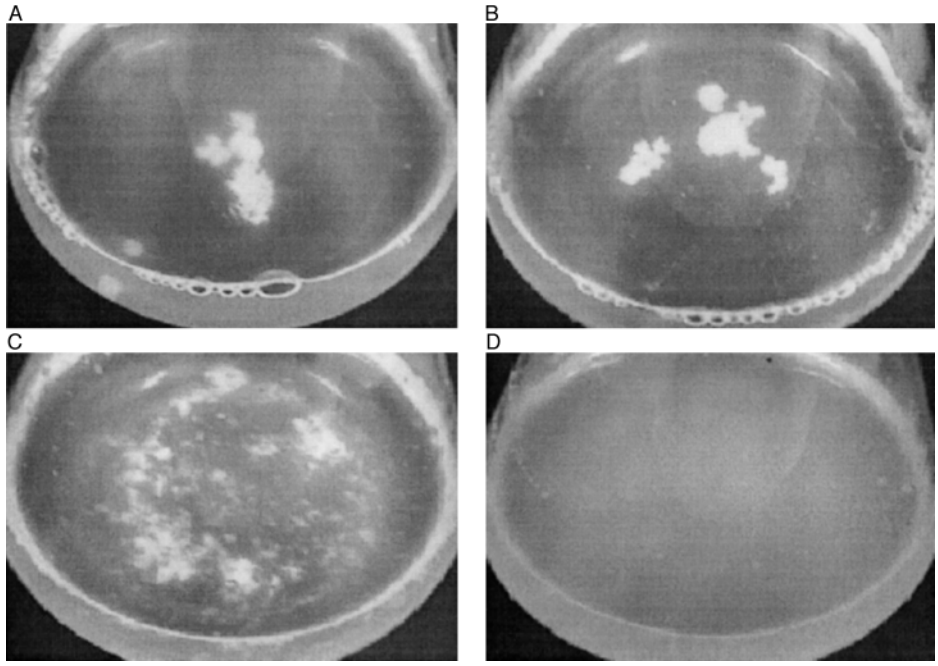


**Figure 4** Cell morphology of *P. putida* CP1 following 48 h growth on (A) 1.56 mM 2-chlorophenol, (B) 0.78 mM 3-chlorophenol, (C) 2.34 mM 4-chlorophenol and (D) 8.5 mM phenol.

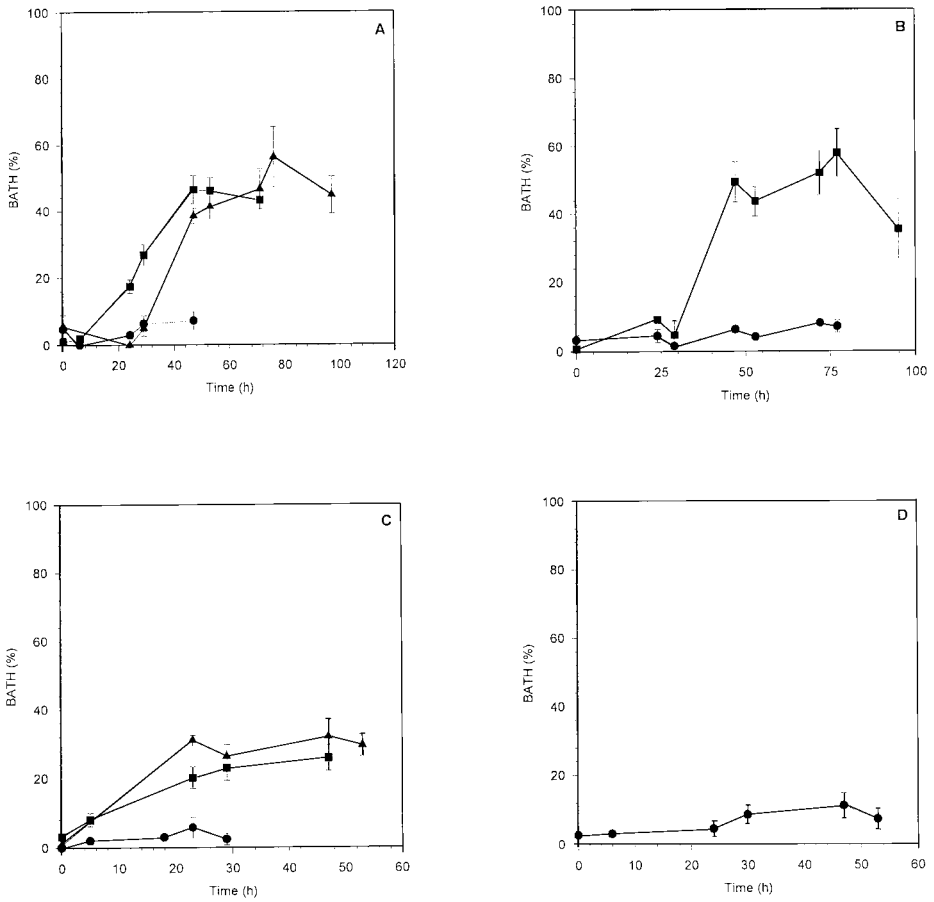


**Figure 5** Hydrophobicity of *P. putida* CP1 during growth on (A) 2-chlorophenol: (●) 0.39 mM, (■) 0.78 mM and (▲) 1.56 mM; (B) 3-chlorophenol: (●) 0.39 mM and (■) 0.78 mM; (C) 4-chlorophenol: (●) 0.78 mM, (■) 1.56 mM and (▲) 2.34 mM and (D) phenol; (●) 8.5 mM.

3- and 4-chlorocatechol at the previously mentioned concentrations or during growth of the organism in nutrient broth.

### *Hydrophobicity of P. putida CP1 during growth on mono-chlorophenols and phenol*

The effect of mono-chlorophenols and phenol on cell surface hydrophobicity during growth was investigated. When *P. putida* CP1 was grown on the mono-chlorophenols at lower concentrations where no visible clumping was observed, 0.39 mM 2- and 3-chlorophenol and 0.78 mM 4-chlorophenol, the BATH assay gave low hydrophobicity readings — less than 10% (Figure 5A–C). Similarly, when *P. putida* CP1 was grown on 8.5 mM phenol, the BATH assay gave a maximum hydrophobicity of 11% (Figure 5D). Growth in nutrient broth resulted in hydrophobicities less than 6%. However, when *P. putida* CP1 was grown on higher concentrations of the mono-chlorophenols, which led to visible clumping in the culture medium, the resultant biomass was more hydrophobic. Increasing the concentration of 2-chlorophenol from 0.39 to 0.78 mM caused the maximum hydrophobicity to rise to 46%, while a further rise in 2-chlorophenol concentration to 1.56 mM increased maximum hydrophobicity to 56% (Figure 5A). A rise in 3-chlorophenol concentration from 0.39 to 0.78 mM resulted in the biomass hydrophobicity increasing to 58% (Figure 5B). This pattern was also seen when the concentration of 4-chlorophenol was increased from 0.78 to 1.56 mM and finally to 2.36 mM, which led to the maximum hydrophobicities increasing to 23% and 32% respectively (Figure 5C). At the single concentration of 0.78 mM, the hydrophobicity was greatest for 3-chlorophenol (58%), followed by 2-chlorophenol (46%) and finally 4-chlorophenol (6%), the reverse of the ability of *P. putida* CP1 to degrade the mono-chlorophenols.

## Discussion

A bacterial isolate identified as a *P. putida* strain and designated the code CP1 was shown to be capable of degrading all three mono-chlorophenol isomers, a phenomenon rarely reported previously. Complete degradation of 2-, 3-, and 4-chlorophenol at concentration of 0.08 mM was demonstrated by immobilised cells of *P. testosteroni* using the *ortho*-cleavage pathway [22]. Only *P. pickettii* LD1 (now *Ralstonia pickettii*) and *Rhodococcus opacus* GM-14 have been reported to degrade all three mono-chlorophenols to completion at concentrations comparable to those degraded by *P. putida* CP1 [15,36]. *P. pickettii* LD1 degraded 1.51 mM 2-chlorophenol, 0.57 mM 3-chlorophenol and 0.75 mM 4-chlorophenol, while *Rh. opacus* GM-14 degraded 1.95 mM 2-/4-chlorophenol and 0.78 mM 3-chlorophenol.

Phenol is more easily degraded than mono-chlorophenols because the substitution of mono-chlorophenols with a chloride electron-withdrawing group diminishes enzymatic transformation and deactivates electrophilic substitution. The differences between the degradation of the mono-chlorophenols result from the

differing positions of the chloride atom on the aromatic ring which, coupled with the electron-accelerating effect of the initial hydroxyl group, affects the introduction of a second hydroxyl group onto the aromatic ring. Due to the competing electron-accelerating/decelerating effects, the theoretical order of degradability should be 4-chlorophenol>2-chlorophenol>3-chlorophenol [25] — the order that was observed during the degradation of the mono-chlorophenols by *P. putida* CP1.

Complete degradation of 4-chlorophenol by the *meta*-cleavage pathway has been demonstrated [13,18,33]. Complete degradation of 2- and 3-chlorophenol using the *meta*-cleavage pathway has not yet been demonstrated, although the degradation of 3-chlorobenzoate *via* a *meta*-cleavage pathway has been demonstrated [23]. Successful metabolism of all three mono-chlorophenols appears to require the *ortho*-cleavage pathway. Assays of the key enzymes involved in the ring cleavage of chloroaromatics, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase indicated that degradation of the mono-chlorophenols and phenol by *P. putida* CP1 was *via* the *ortho*-cleavage pathway. Cells grown on phenol displayed *ortho*-activities mainly towards catechol, while cells grown on the mono-chlorophenols displayed similar activities with both catechol and chlorocatechol. This suggests that *P. putida* CP1 possesses two different *ortho*-cleavage enzymes, a catechol 1,2-dioxygenase capable of metabolising catechol (*ortho*-I activity) and a chlorocatechol 1,2-dioxygenase with low substrate specificity capable of metabolising both catechol and chlorocatechols (*ortho*-II activity) similar to the chlorocatechol-degrading organism, *Pseudomonas* sp. B13 [11].

*P. putida* CP1 was also shown to be capable of degrading 3- and 4-chlorocatechol, known intermediates of chlorophenol degradation. 3-Chlorocatechol metabolism was more rapid than that observed during 4-chlorocatechol degradation, indicating a higher affinity of chlorocatechol 1,2-dioxygenase for 3-chlorocatechol than 4-chlorocatechol. Degradation of 4-chlorophenol, which occurs *via* 4-chlorocatechol, was at higher rates than the degradation of 2- or 3-chlorophenol, which is degraded *via* 3-chlorocatechol. This suggests that the initial step in degradation of chlorophenols, hydroxylation of chlorophenols to chlorocatechols, was the rate-limiting step in degradation of chlorophenols by *P. putida* CP1. This was confirmed by incubation of lower concentrations of 3- and 4-chlorocatechol (0.13 mM), which were metabolised almost immediately without any lag. Metabolism of 0.13 and 0.35 mM 3-/4-chlorocatechol resulted in stoichiometric releases of chloride indicating complete degradation. During the degradation of 0.69 mM 3-/4-chlorocatechol by *P. putida* CP1, a brown colour developed in the culture medium, presumably due to the formation of chlorocatechol polymers as a result of autooxidation. In this instance, *P. putida* CP1 appeared unable to metabolise the chlorocatechol polymers, as chloride releases following degradation of 0.69 mM 3-/4-chlorocatechol were not stoichiometric.

When *P. putida* CP1 was incubated in the presence of higher concentrations of mono-chlorophenol, $\geq$1.56 mM 4-chlorophenol and $\geq$0.78 mM 2- and 3-chlorophenol, it formed large clumps of cells in the culture medium. At these concentrations, incubation with the mono-chlorophenols resulted in decreases in dry weights. Although *P. putida* CP1 was capable of mono-chlorophenol degradation, there was a net decrease in cell biomass indicating a toxic effect of the mono-chlorophenols at increased concentrations. Degradation of mono-chlorophenols by *Ra. pickettii* [15] and *Rh. opacus* GM-14 [36] resulted in increases in cell biomass corresponding to degradation of the mono-chlorophenols as was observed during the metabolism of lower concentrations of mono-chlorophenols, 0.78 mM 4-chlorophenol and 0.39 mM 2- and 3-chlorophenol and phenol by *P. putida* CP1. At these lower concentrations of mono-chlorophenol and phenol and during growth of the organism on relatively low concentrations of 3-/4-chlorocatechol and nutrient broth, no aggregation of the organism was observed. This suggested that clumping may have resulted from toxic effects of mono-chlorophenols. Hydrophobicity, measured by bacterial adherence to *n*-octane, was used to study clumping of *P. putida* CP1. Bacterial hydrophobicity has been used to investigate the adhesion of microorganisms to many surfaces, including the co-adhesion of bacteria [12], and it has also been used to study substrate-dependent clumping of a *Pseudomonas* strain [29]. The autoaggregating mutant *Ra. eutropha*-like strain A3 was more hydrophobic than its nonaggregating parent, *Ra. eutropha*-like strain AE815 [8]. *P. putida* CP1 cells grown on higher concentrations of mono-chlorophenol were also more hydrophobic than those grown on lower concentrations. When *P. putida* CP1 was grown on phenol at concentrations as high as 8.5 mM, cells were not hydrophobic, and no aggregation was observed, also suggesting a link between substrate toxicity and autoaggregation. Growth of *P. putida* CP1 on 4-chlorophenol, the least toxic of the mono-chlorophenols, resulted in formation of more numerous but smaller clumps than were formed during degradation of the more toxic 2- and 3-chlorophenol by *P. putida* CP1. Hydrophobicity assays showed the cells grown on 2- and 3-chlorophenol to be more hydrophobic than those grown on 4-chlorophenol.

The results of experiments described in this study suggest that *P. putida* CP1 flocculates as a result of chemical stress, as clumps formed in the presence of higher concentrations of mono-chlorophenols. Bacterial aggregation as a result of environmental stress has been described [5,7]. *Comamonas testosteroni* A20 displayed phenotypic instability resulting in differing cell morphologies when the organism was grown under differing conditions. *C. testosteroni* A20 formed mucoid cells when grown under favourable conditions. However, when it was grown under unfavourable conditions, there was a phenotypic shift from mucoid colony-forming cells to nonmucoid colony-forming cells [5]. Under favourable conditions where mucoid cells were formed, loose associations between *C. testosteroni* A20 cells resulted, while under unfavourable conditions where nonmucoid cells were formed, dense flocs resulted. Unfavourable conditions under which *C. testosteroni* A20 formed a nonmucoid phenotype included the presence of hydrogen peroxide, sodium lauryl sulfate and starvation periods. Nonmucoid cells were also formed in the presence of acute heat shock, reactive oxidative intermediates and sublethal doses of visible light [7]. As was observed during experiments described in this paper, nonmucoid cells capable of flocculation were more hydrophobic than mucoid cells. It was proposed that nonmucoid cells formed under conditions of environmental stress were more stress-resistant than mucoid cells as the flocs provided protection against cell-penetrating chemicals. As chlorophenol removal at higher substrate concentrations did not begin until *P. putida* CP1 had formed clumps of cells, it is possible that the ability of *P. putida* CP1 to clump conferred a protective advantage, allowing for chlorophenol degradation to occur.

This study demonstrated the complete degradation of all three mono-chlorophenols *via* an *ortho*-cleavage pathway by a flocculating organism, *P. putida* CP1. Floc-forming bacteria

capable of degrading xenobiotics are rare as demonstrated by the construction of a genetically engineered flocculating microorganism capable of degrading phenol [32]. This involved introduction of plasmid pS10-45, responsible for degradation of phenol, into the naturally flocculating organism, *Sphingomonas paucimobilis* 551. Degradative genes were introduced into a floc-forming bacterium as flocculation requires multiple genes, and hence, their cloning into a degradative organism would be more difficult. Naturally occurring floc-forming organisms capable of degrading xenobiotics have been described. An activated sludge isolate, strain AS2, capable of degrading 3-chlorobenzoate was described as forming a mucoid mass when grown in shaken liquid culture. The mass settled readily when shaking stopped and produced mucoid colonies on solid media [24]. Another activated sludge isolate, *C. testosteroni* I2, capable of degrading 3-chloroaniline displayed unstable colony morphology on agar plates, suggesting the production of exopolysaccharides, and tended to form clusters within sludge flocs [4]. Such of these floc-forming bacteria has each been used successfully to augment activated sludge for degradation of xenobiotics. The flocculating nature of these bacteria may have been an advantage in bioaugmentation as augmentation of bacteria that may be incorporated into sludge flocs with flocculating bacteria may improve the chances of successful bioaugmentation. Such organisms may stay in activated sludge units longer, helping to maintain the degradative capacity in the sludge for longer periods. *P. putida* CP1 has been used to successfully augment a commercial mixed culture for degradation of 2-chlorophenol [14]. The ability of the organism to express its degradative capabilities, in addition to its ability to flocculate, shows the potential of the *P. putida* CP1 strain to be used in augmentation of activated sludges for treatment of chlorinated aromatics.

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