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Two Different Pathways (a Chlorocatechol and a Hydroquinone Pathway) for the 4-Chlorophenol Degradation in Two Isolated Bacterial Strains

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Two isolated strains, *Comamonas testosteroni* CPW301 and *Arthrobacter ureafaciens* CPR706, were able to use 4-chlorophenol (4-CP) as a sole carbon and energy source. CPW301 was found to degrade 4-CP via a *meta*-cleavage pathway in which the chloro-substituent was eliminated even when 4-chlorocatechol was cleaved by the catechol 2,3-dioxygenase. In contrast, CPR706 removed chloride from 4-CP prior to the ring-fission reaction, producing hydroquinone as a transient intermediate during 4-CP degradation. CPR706 exhibited much higher tolerance for 4-CP than CPW301, which was indicated by the maximum degradable concentration and degradation rate.

Polychlorinated phenols are common pollutants because of their extensive use as biocides and organic precursors in the synthesis of chlorophenoxyacetate herbicides. Under anaerobic conditions, they are frequently transformed by reductive dechlorination to various lower chlorinated phenols including 4-chlorophenol (4-CP) and phenol (7, 8, 16, 22).

Several aerobic bacterial strains have been reported to degrade 4-CP completely via an *ortho*-cleavage pathway (5, 13, 20) or a *meta*-cleavage pathway (12). In both pathways, 4-CP is hydroxylated to form 4-chlorocatechol which is subjected to an intra-diol cleavage before the chloro-substituent is removed. In a practical sense, these pathways may be disadvantageous for the 4-CP degradation because the chlorocatechol and chlorinated metabolites are frequently inhibitory to cell growth and the degradation processes (6, 10).

In the present study, we will describe the initial metabolic pathway for 4-CP degradation in two isolated bacterial strains, *Arthrobacter ureafaciens* CPR706 and *Comamonas testosteroni* CPW301. We will also discuss chloride elimination at the first step of 4-CP degradation by CPR706 and its role in 4-CP metabolism.

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MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Two 4-CP degrading strains were isolated by a selective enrichment procedure, and identified as *Arthrobacter ureafaciens* CPR706 and *Comamonas testosteroni* CPW301, respectively (3).

The isolates were cultivated in a minimum salts medium (MSM) composed of the following components (per liter): 1 g K_2HPO_4, 0.6 g NaH_2PO_4, 1 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.002% yeast extract, and 1 ml trace element solution (14). All batch cultures were carried out in 500-ml Erlenmeyer flasks containing 50 ml of liquid MSM in a shaking incubator (120 rpm) at 30°C. Fresh colonies grown on Bacto-nutrient agar plates containing 50 mg/l 4-CP (Difco Laboratories, Detroit, MI, U.S.A.) were suspended and shaken in MSM overnight (about 16 h) before 4-CP (Aldrich-Chemical Co., Ltd., Milwaukee, WI, U.S.A.) was added as a carbon source.

For resting cell experiments, cells grown on 4-CP were twice washed with 50 mM phosphate buffer (pH 7.5) and resuspended in the same buffer. Suitable testing carbons were supplemented with the cell suspension, and incubated at 120 rpm and 30°C.

Isolation and Identification of Metabolites

Metabolite formation during 4-CP degradation was

monitored by scanning a culture medium sample with a spectrophotometer (Beckman, Model DU-68, Fullerton, CA, U.S.A.) in the UV range. When detectable levels of metabolites appeared, the supernatant of the culture sample was acidified (pH 2.0) with HCl and extracted with ethyl acetate. The extracted organic layer was dried over sodium sulfate beads and then evaporated to a small volume. The extracted metabolites were treated with *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (TMS) to generate TMS-derivatives, and analyzed with gas chromatography-mass spectrometry (Fisons, Altrincham, England). The ion energy in the mass spectrometry was 70 eV. The oven temperature was kept at 100°C for 2 min and then increased to 280°C at the rate of 20°C per min. A fused-silica capillary column was coated with SE-30-cross-linked methylsilicone (25 m × 0.25 mm I.D.).

Analytical Methods

Cell growth was determined by measuring the increase in cell turbidity at 600 nm with a spectrophotometer (Beckman, Model DU-68, Fullerton, CA, U.S.A.). The absorbance was converted to dry weight by using a standard curve. The specific 4-CP degradation rate was calculated as the change of 4-CP concentration divided by the time required and by an average cell concentration, dS/(dt X). The specific growth rate was expressed as the change in cell mass divided by time required and by cell mass at that time, dX/(dt X). Chloride ion concentration was determined by using a pH meter (Orion, no. 701A, Boston, MA, U.S.A.) with a chloride specific electrode. The standard chloride concentration was calibrated with NaCl in minimal salts medium before measurement. The concentrations of 4-CP in the culture fluid were determined with a reverse-phase HPLC (Waters Associates, MA, U.S.A.) equipped with a Nova pack C_{18} column (3.9 × 150 mm) and a UV detector at 280 nm. The mobile phase was composed of methanol-water-acetic acid (100 : 100 : 2).

Measurement of Enzyme Activities

Cells grown on 4-CP were washed with 50 mM Tris-HCl buffer (pH 7.5) and resuspended in the same buffer containing 1 mM ascorbic acid. The cells were broken by means of ultrasonic treatment in an ice bath and centrifuged at 20,000 × g for 30 min at 4°C. The supernatant was used as a crude enzyme extract immediately afterward. Catechol 2,3-dioxygenase activity was determined by the method described by Asturias and Timmis (2). Catechol 1,2-dioxygenase activity was measured according to the method described by Dorn and Knackmuss (9). Hydroquinone oxgenase activity was determined by measuring the formation of ring-fission product at 320 nm as described by Spain and Gibson (18).

One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per min in 1 ml reaction mixture. Specific activities were expressed as units per mg of protein. Protein concentration was measured by the method of Lowry *et al.* (15) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Dechlorination and Metabolite Accumulation in Batch Cultures of Two Isolates

Fig. 1 shows the accumulation of intermediates and chloride ions during 4-CP degradation in batch cultivation of each isolate. In the process of 4-CP degradation, the CPW301 culture broth became notably greenish-yellow in color, exhibiting an absorption maximum at 379 nm (Fig. 1A). This observation indicates the excretion of a *meta*-cleavage intermediate of 4-chlorocatechol. After the complete depletion of 4-CP, the colored intermediate slowly disappeared. Almost equimolar amount (above 96.5%) of chloride was released into the culture medium when 4-CP and its intermediate completely disappeared, indicating the complete break-down of both compounds.

CPR706 also completely degraded the supplemented 4-CP, releasing the stoichiometric amount of chloride ions

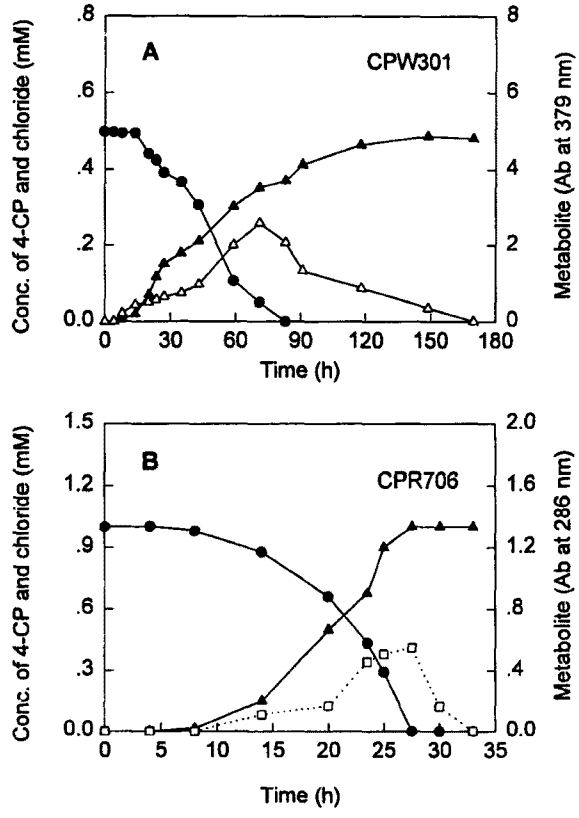

Fig. 1. The release of chloride and metabolites during the 4-CP degradation by two isolates.

Symbols: ●, 4-CP; ▲, chloride; △, a metabolite formed in CPW301 culture medium; □, a metabolite formed in CPR706 culture medium.

into the culture medium (Fig. 1B). During the 4-CP degradation, a metabolite (an absorption maximum at 286 nm) transiently appeared in the culture broth and disappeared after the complete depletion of 4-CP. In contrast to the case of CPW301 culture, the disappearance of the metabolite did not accompany the further release of chloride. This result suggests that this intermediate does not contain any chloride ion.

Identification of Intermediate Metabolites

In order to identify metabolites produced by 4-CP degradation, each culture broth was extracted with ethyl acetate and analyzed with gas chromatography-mass spectrometry. Fig. 2A shows the mass spectrum of a major metabolite produced during the 4-CP degradation by CPW301. Its primary fragment ions are found at *m/z* 305 ($M-CH_3$), 285 (M-Cl), 231 (M-OTMS), 203 (M-COOTMS), and 167 (M-OTMS-Cl-CHO). The characteristic M/M +2 ratio of 3 : 1 resulting from the $^{35}Cl/^{37}Cl$ isotope ratio at *m/z* 305, 231, and 203 shows the presence of a single Cl atom in this intermediate. This mass spectrum is very consistent with that previously reported for 5-chloro-2-hydroxy muconic semialdehyde (19).

Fig. 2B shows the mass spectrum of a major intermediate extracted from CPR706 culture medium. Its primary fragment ion at *m/z* 254 indicates that its molecular weight is 110. No evidence for the presence of chloride in this intermediate was found in this spectrum. This metabolite was identified as hydroquinone by comparison with that of an authentic compound.

Degradability of 4-Substituted Phenols and Metabolites by CPR706

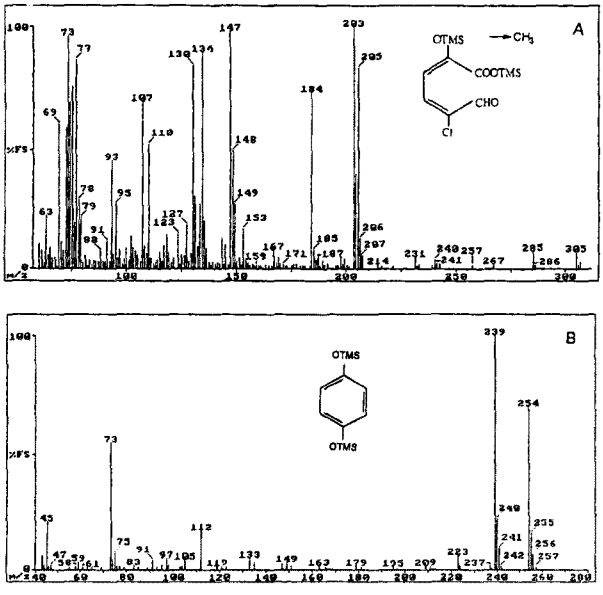

Fig. 2. The mass spectrum of the metabolite formed from 4-CP degradation by CPW301 (A) and by CPR706 (B).

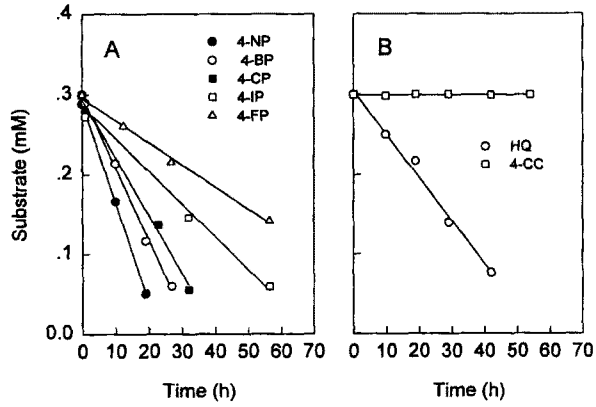

Fig. 3. The degradation of 4-substituted phenols (A) and metabolites (B) by the resting cells of CPR706.
The cell concentration in reaction buffer was 0.5 g dry weight per liter.

To see the metabolic properties of CPR706, resting cells grown on 4-CP were incubated with several 4-substituted phenols and possible 4-CP metabolites. As shown in Fig. 3A, the resting cells degraded all 4-substituted phenols tested without a lag phase, during which hydroquinone appeared as in the case of 4-CP degradation (data not shown). This result indicates that they were degraded by the same enzyme system catalyzing 4-CP degradation. No phenols substituted in *ortho-* or *meta*-position were degraded by the resting cells (data not shown), indicating that their metabolic pathways were different from those for 4-CP.

Fig. 3B shows that hydroquinone was successfully degraded by the resting cells, whereas 4-chlorocatechol was not degraded. These results suggest that hydroquinone is an apparent intermediate occurring during the catabolic pathway of 4-CP, but chlorocatechol is not.

It is noteworthy that CPR706 replaces chloride with the incoming hydroxyl group to form hydroquinone before the benzene ring is cleaved (Fig. 4b). All previous

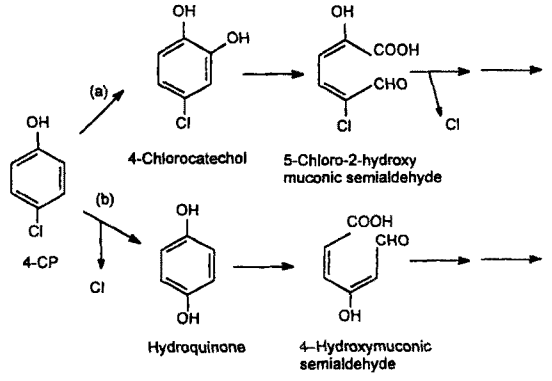

Fig. 4. Two different initial pathways of 4-CP degradation: (a) *meta*-cleavage pathway and (b) hydroquinone pathway.

studies have shown that the degradation of mono-chlorophenols are initiated by hydroxylation at the second carbon position to form chlorocatechols (Fig. 4a), in which the chloro-substituent is removed even when the benzene ring is cleaved (9, 11, 13).

Ring Cleavage Enzymes

In order to find the initial pathway for the 4-CP degradation by two isolates, the activities of key enzymes catalyzing ring cleavage were assayed by using catechols or hydroquinone as substrates (Table 1). The crude extract from CPW301 exhibited high catechol 2,3-dioxygenase activity, whereas it did not show any activity of catechol 1,2-dioxygenase or hydroquinone oxygenase. These results indicate that CPW301 can metabolize 4-CP via a *meta*-cleavage pathway as postulated in Fig. 4a. The *meta*-cleavage pathway of 4-CP had been known not to support cell growth due to the formation of toxic metabolites (6, 21). Recently, however, a few strains were reported to metabolize 4-CP via the *meta*-cleavage pathway (1, 12).

On the other hand, the crude enzyme extract from CPR706 revealed hydroquinone oxygenase activity, but did not show any activity of catechol 2,3-dioxygenase or catechol 1,2-dioxygenase. We previously demonstrated that hydroquinone was enzymatically oxidized to 4-hydroxymuconic semialdehyde (4) as illustrated in Fig. 4b.

Growth and Degradation Rate in Various Initial Concentrations of 4-CP

In the previous sections, two strains were demonstrated to metabolize 4-CP through different metabolic pathways. One of the greatest distinctions between these two pathways lays in the mode of dechlorination: CPR 706 eliminated chloro-substituents before the ring-fission reaction, whereas CPW301 did this after the ring-cleavage. Frequently, chlorinated metabolites may affect cell growth and degradation processes due to the fact that chlorocatechol can inactivate ring-fission dioxygenases because it forms a chelate with the ionic enzyme cofactor (10), and that (2) the ring fission products of chlorocatechol can also decrease enzymatic transformation rates and deactivate electrophilic substitutions during the degradation process because of the electron withdrawing effect of the chloro-substituent (17). Thus, the 4-CP degradation by CPR706 may be advantageous over that of CPW301 because the toxic chloro-substituent is eliminated at the first step of the degradation pathway. In order to confirm such effects, we investigated the growth and degradation patterns of two isolates in batch cultures with various initial 4-CP concentrations.

As shown in Fig. 5A and 5B, CPR706 exhibited about 3.5 folds higher specific growth rate and about 2 folds higher specific 4-CP degradation rate than CPW301. It is also noteworthy that CPR706 exhibited much higher tolerance for 4-CP than CPW301, as demonstrated by the maximum 4-CP concentration for CPR706 or CPW 301 to degrade (1.6 mM for CPR706 and 0.8 mM for CPW301). This tolerance of CPR706 for 4-CP is also higher than that of a typical 4-CP degrading strain (with another chlorocatechol pathway, *ortho* cleavage pathway), *Alcaligenes* sp. A7-2 (5). Though we cannot compare the degradation rates of other strains to show which pathway is more effective in degrading 4-CP, the high 4-CP tolerance and 4-CP degradation rates induced by

Table 1. Specific enzyme activities of CPW 301 and CPR706 after induction with 4-CP.

Enzyme assayed and assay substrate	Specific enzyme activity of	
	CPW301	CPR706
Catechol 2,3-dioxygenase:		
Catechol	0.17	0.00
4-Chlorocatechol	0.02	0.00
Catechol 1,2-dioxygenase:		
Catechol	0.00	0.00
4-Chlorocatechol	0.00	0.00
Hydroquinone oxygenase:		
Hydroquinone	0.00	1.02

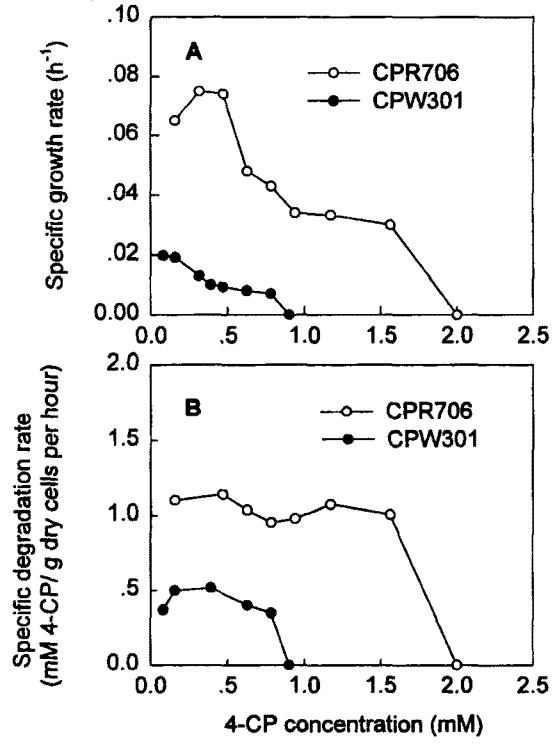

Fig. 5. The effects of initial 4-CP concentrations on the specific growth (A) and the specific 4-CP degradation (B) rates by CPW301 and CPR706.

CPR706 are thought to be attributable to the reduced inhibition and the elimination of chloro-substituent in the hydroquinone pathway.

In this study, our strains were found to degrade 4-CP through unusual metabolic pathways, a *meta*-cleavage and a hydroquinone pathway. Particularly, the hydroquinone pathway is quite different from the typical mechanism of 4-CP degradation (an *ortho*-cleavage pathway). These strains may be useful for comparative study of the variety of bacterial metabolisms in degradation of mono- or di-chlorophenols.

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