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BIODEGRADATION OF TRIIODOPHENOL BY CELL-FREE EXTRACTS OF A PENTACHLOROPHENOL-DEGRADING *FLAVOBACTERIUM* SP.[@]

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Pentachlorophenol (PCP) degrading *Flavobacterium* sp. ATCC 39723 was found to degrade other polyhalogenated phenolic compounds, including triiodophenol, tribromophenol, and trichlorophenol. Each compound was able to induce the degradation of the other compounds. A PCP$^-$ *Flavobacterium* sp. mutant, F-2, was unable to degrade any of the halogenated compounds. The results suggest that all of the polyhalogenated phenols were degraded by the same enzyme system. This observation led us to exploit the sensitive leuco crystal violet assay, which measures the iodide released from triiodophenol. Cell free extracts from PCP-induced cells were able to release iodide from triiodophenol. The reaction required NADPH and oxygen.

Several organisms which mineralize pentachlorophenol (PCP) have been isolated (1,3,7,10,11,13,14), one of which is a *Flavobacterium* sp. (7). This *Flavobacterium* sp. also degrades several other polychlorinated phenols, including 2,4,6-trichlorophenol (TCP) (12). Degradation of polyiodophenols or polybromophenols has not been previously reported for PCP-degrading organisms. Here we report the degradation of 2,4,6-triiodophenol (TIP) and 2,4,6-tribromophenol (TBP) by the PCP-degrading *Flavobacterium* sp. ATCC 39723.

Although dehalogenation of polyhalogenated phenols is essential for degradation of such compounds, progress in understanding the enzymology and genetics of dehalogenation has been slow. The lack of a sensitive assay has been one barrier. After demonstrating that iodide is released from TIP by the PCP-degrading *Flavobacterium*, we applied the sensitive leuco crystal violet assay to measure the iodide released from TIP in cell-free extract assays.

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MATERIALS AND METHODS

Culture and Whole-cell Assay Conditions. *Flavobacterium* sp. ATCC strain 39723 was cultured in mineral medium containing 4 g/liter of sodium glutamate as carbon source (6). PCP, TBP, TCP, and TIP degradation was induced by adding 40 ppm of the halogenated compound to early log phase cultures of the bacterium (7). Bacterial cells were collected by centrifugation in a microcentrifuge for 2 min at room temperature. The concentrations of PCP, TCP, TIP, and TBP in the supernatant were measured spectrophotometrically at 320 nm. Iodide concentration was measured colorimetrically by the leuco crystal violet method (2) with minor modifications. We proportionally reduced the assay from 100 ml to 1 ml. The method was based on the following principle: iodide is selectively oxidized to iodine by potassium peroxymonosulfate. The iodine produced reacts instantaneously with the colorless leuco crystal violet to produce the highly colored crystal violet dye. The color is stable and measured spectrophotometrically at a wavelength of 592 nm.

Cell-free Extract Preparation. Cell-free extracts were obtained by suspending 1 g of PCP-induced cells (wet weight) in 5 ml of 20 mM Tris (pH 8.0) and passing twice through an Aminco Model 4-3398 French Pressure cell (7,000 p.s.i.). A protein inhibitor, phenylmethylsulfonyl fluoride (Sigma), was added to the cell suspension to a final concentration of 5 mM just before breaking the cells. Unbroken cells were removed by centrifugation at 10,000 x *g* for 10 min. Protamine sulfate (Sigma) was added to a final concentration of 1 mg/ml, and the sample was centrifuged at 10,000 x *g* for 10 min to remove nucleic acid-protamine complex. The supernatant, free of intact cells, were used for enzyme-activity assays.

Enzyme Activity of Cell-free Extracts. The reaction mixture contained 1.2 mg of protein, 100 μM TIP, 50 mM Tris-acetate buffer, 10 mM $MgSO_4$, and, if included, 0.5% Trition X-100 (Sigma), atmospheric oxygen, and 100 μM NADPH in a total volume of 1 ml. The samples were incubated at 24°C in the dark overnight. A standard curve of iodide was made in the same reaction mixture without TIP.
Proteins were removed from the completed assays by acidifying 0.25 ml samples with citric buffer (0.05 ml), boiling for 3 min, and centrifuging for 2 min at 15,900 x *g*. The supernatant (0.25 ml) was diluted to 0.5 ml with distilled water, and 10 μl of potassium peroxymonosulfate solution and 10 μl of leuco crystal violet solution were added for measurement of free iodide. The O_2-free assay was performed in a serum tube. Helium gas was passed through the assay mixture without TIP for 5 min, TIP was added, and the tube was sealed with a rubber stopper. At the end of the assay, the tube was immersed into a boiling water bath for 3 min and iodide release was assayed.

RESULTS AND DISCUSSION

Degradation of TIP, TBP, and PCP was induced in *Flavobacterium* sp. ATCC 39723 in approximately 40 min by each of the respective substrates (Fig. 1). PCP added at 180 min to those cultures previously induced with TIP or TBP was also degraded without a lag period. This phenomenon suggested that one enzyme system was responsible for degradation of all three halogenated compounds. The ability of one enzyme system to remove different halogens from otherwise similar substrates

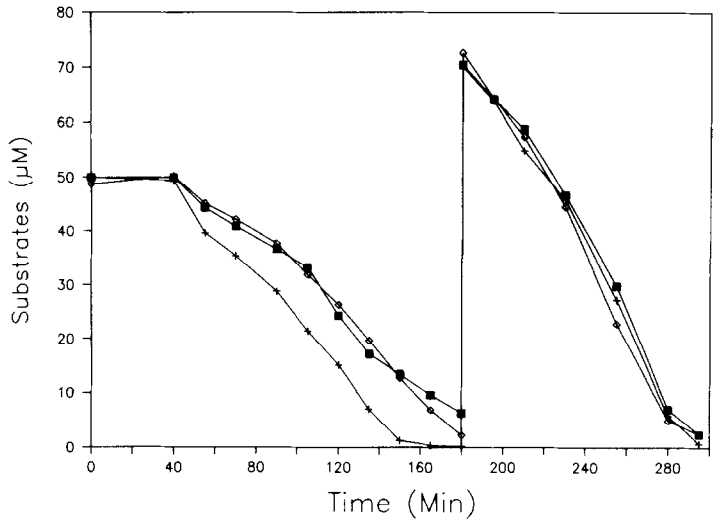

Fig. 1. PCP-degradation by *Flavobacterium* sp. ATCC 39723 induced with TIP (■), TBP (+), or PCP (◇). PCP concentration was monitored using absorbance at 320 nm. PCP (70 ppm) was added to all cultures at 180 min.

is not without precedent. A dioxygenase which dechlorinates 4-chlorophenylacetate to produce 3,4-dihydroxyphenylacetate also uses 4-fluorophenylacetate and 4-bromophenylacetate as substrate (5,6). Most chloroalkane and chloroalkanoic acid dehalogenases also work on more than one halogen substitution (9). Furthermore, the PCP⁻ *Flavobacterium* sp. mutant F-2, isolated for its inability to degrade PCP (11), was also unable to degrade TIP, TBP, or TCP.

The *Flavobacterium* sp. ATCC 39723 TIP-induced cells degraded PCP, TCP, TBP and TIP at different rates (Fig. 2). The cells degraded TCP and TBP most rapidly, followed by PCP, and finally TIP. TCP is less chlorinated than PCP and faster degradation was expected. Although TIP is less halogenated than PCP, iodine substitution may interfere with enzyme binding as a consequence of the halogen size. Bromine, having a smaller ionic radius may not interfere with enzyme binding, as it was degraded at a similar rate to TCP.

Fig. 2 shows that iodide production occurred at a rate expected by an equivalent amount of TIP degradation. Three iodides were released for every TIP molecule degraded. Approximately 160 μmol of iodide was released and 53 μmol of TIP was degraded after 90 min of incubation. The leuco crystal violet assay can detect as little as 5 μmoles of iodide. Since the assay is very quick, accurate, and sensitive, iodide released from TIP can be used as a dehalogenating enzyme assay.

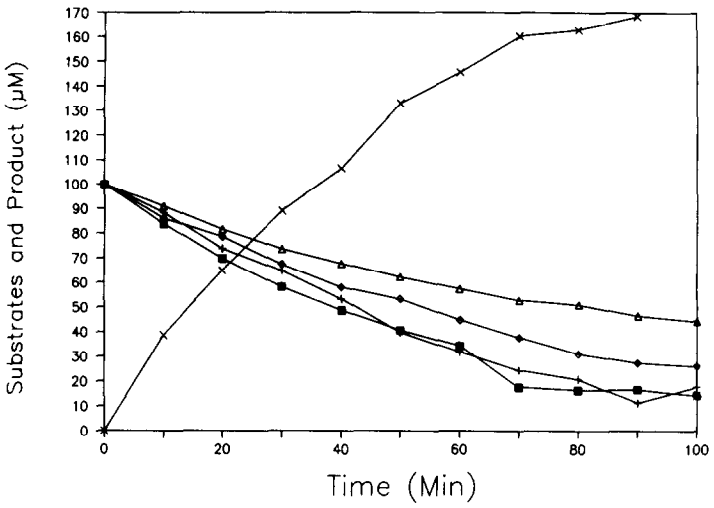

Fig. 2. Degradation of polyhalogenated phenols by TIP-induced *Flavobacterium* sp. strain ATCC 39723 cells. After TIP-induction, the cells were harvested by centrifugation, resuspended in 100 mM Tris buffer, pH 8.0, adjusted to an optical density $A_{420} = 1.00$, and supplied with 100 μM of TCP, TBP, TIP, or PCP. Concentrations of TCP (■), TBP (+), TIP (△), or PCP (◇) were monitored spectrophotometrically and iodide (×) concentration was measured by the leuco crystal violet assay.

The most convenient method to measure PCP or TIP concentration is spectrophotometrically at 320 nm. However, cofactors, such as NADPH, NADH and FAD which may be added to catalyze enzyme reactions, can interfere with this method and enzymatic reaction end-products may also absorb at 320 nm. Another simple method of monitoring PCP-degradation is the use of a chlorine electrode; however, the presence of chlorine in the assay solution can produce high background concentrations. Other methods, including HPLC and GC-MS, have considerable cost and take 10 min or longer for each sample and therefore are not practical for routine screening of enzyme degrading reactions of polyhalogenated phenols. It is possible to screen many samples in a short time for the presence of dehalogenating enzymes, using the simple and sensitive leuco crystal violet method of measuring iodide concentration.

Our previous attempts to demonstrate dehalogenase activity with *Flavobacterium* sp. cell-free extracts had been unsuccessful. We hypothesized that PCP-degrading activity within the cell was membrane associated, and required both membrane bound and soluble proteins and therefore, when the cells were broken, the soluble proteins could not orient correctly with membrane proteins. We further

hypothesized that once the membrane vesicles were dissolved with non-ionic detergents, the freed membrane bound proteins and soluble proteins could reassociate and restore some of the dehalogenating activity. In fact, the addition of Triton X-100 to the assay mixture at a final concentration of 0.5% resulted in a detectable level of released iodide. TIP concentration, as analyzed by HPLC using an acetonitrile gradient, indicated approximately 12 μM of TIP disappeared from the sample which released 42 μM iodide. No accumulated intermediate was detected suggesting that TIP was completely dehalogenated. The activity required Triton X-100, NADPH, and oxygen. NADH cannot replace NADPH. Incubation of the assay mixture for at least 4 hours was required for detection of any iodide released, which indicated the cell-free enzyme activity was relatively low. The control assay reactions containing boiled, inactivated extracts or no cell-free extracts did not generate any free iodide. These results strongly suggest the release of iodide was an enzymatic reaction.

Our results agree with the data reported by Schenk et al. (8). They demonstrated that a sucrose gradient fraction contained enzyme activity which converted PCP to TeCH. The reaction also required NADPH and O_2. They further suggested that the enzyme could be a monooxygenase. Haggblom et al. (4) demonstrated that TeCH was converted to 1,2,4-trihydroxylbenzene by cell-free extracts of a PCP-degrading *Rhodococcus* sp. in the presence of ascorbic acid. However, the mechanism of the conversion is not known. We have not been able to detect the conversion of TeCH by the cell-free extract from *Flavobacterium* sp ATCC 39723.

The low enzyme activity from the cell-free extract could be due to several reasons. First, the conditions we used are not the same as the enzymes' natural environment. Second, there are other enzymes which also effectively oxidized NADPH. The cell-free extract of both PCP-uninduced and PCP-induced cells rapidly oxidized NADH or NADPH with or without TIP or PCP. We demonstrated that NADPH was a required substrate for TIP-degradation. However, the added NADPH was rapidly oxidized by other proteins, so that the amount of NADPH used for TIP-degradation was much smaller than that of NADPH added to the reaction mixture. The level of competition for NADPH by the dehalogenase with other NADPH oxidizing proteins is not clear at this moment. A further possibility is that an additional cofactor is required for TIP-degradation, which is reduced by NADPH first.

In summary, TIP, TBP, TCP, and PCP were degraded by *Flavobacterium* sp. ATCC 39723 by the same induced enzyme system. Each of the halogenated compounds induced the degradation of the other halogenated compounds. We have

used the simple and sensitive leuco crystal violet method to monitor TIP-degradation in both whole cell and cell-free extract assays. Enzymatic activity of TIP-degradation was detected by this method. Currently, we are using this method to isolate the proteins involved in TIP-degradation. The same enzymes may also degrade PCP.

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