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Biodegradation of the Herbicide Bromoxynil (3,5-Dibromo-4-Hydroxybenzonitrile) by Purified Pentachlorophenol Hydroxylase and Whole Cells of *Flavobacterium* sp. Strain ATCC 39723 Is Accompanied by Cyanogenesis†

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A pentachlorophenol (PCP)-degrading *Flavobacterium* sp. (strain ATCC 39723) degraded bromoxynil with the production of bromide and cyanide. No aromatic intermediates were detected in the spent culture fluid. The cyanide produced upon bromoxynil metabolism was inhibitory to the *Flavobacterium* sp. Whole cells degraded PCP more rapidly than they did bromoxynil. Bromoxynil metabolism and PCP metabolism were coinduced, either substrate serving as the inducer. Purified PCP hydroxylase degraded bromoxynil with stoichiometric accumulation of cyanide and without bromide production. A product accumulated which was more hydrophilic than bromoxynil upon high-pressure liquid chromatographic analysis and which, when analyzed by gas chromatography-mass spectrometry, had a mass spectrum consistent with that expected for dibromohydroquinone. PCP hydroxylase consumed NADPH, oxygen, and bromoxynil in a 2:1:1 molar ratio, producing 1 mol of cyanide per mol of bromoxynil degraded. We propose a pathway by which bromoxynil is metabolized by the same enzymes which degrade PCP. The initial step in the pathway is the conversion of bromoxynil to 2,6-dibromohydroquinone by PCP hydroxylase. In addition to its utility for decontaminating PCP-polluted sites, the *Flavobacterium* sp. may be useful for decontaminating bromoxynil spills. This is the first report of cyanide production accompanying the metabolism of a benzonitrile derivative.

Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) is a selective, contact, preemergence herbicide used on a variety of crops in the United States, Canada, and elsewhere. Bromoxynil is an uncoupler of oxidative phosphorylation (5) and has been detected in rivers draining agricultural watersheds (8). Bromoxynil esters contained in various herbicide formulations are rapidly converted to the phenol in wetlands (9). The half-life of bromoxynil in soil is generally several days, and biodegradation is the primary mechanism of dissipation (16). Bromoxynil degradation by a *Flexibacterium* sp. and an isolate of *Klebsiella pneumoniae* subsp. *ozaenae* has been described previously (4, 7, 17). In both cases, metabolism was initiated by hydrolysis of the cyano group to the carboxylate (7, 17).

A *Flavobacterium* sp. isolated from soil (ATCC 39723) mineralizes pentachlorophenol (PCP) and other polyhalophenols (3, 6, 11, 20, 21, 24–27). PCP is an important wood preservative. Soils and water polluted with this compound can be decontaminated by the addition of this organism (3, 6, 26). Polyhalophenol isomers subject to degradation by this organism have in common substitution in the 2 and 6 positions with chlorine, bromine, or iodine atoms (21, 27). PCP metabolism is initiated by the replacement of the *para* chlorine with a hydroxyl group to yield 2,3,5,6-tetrachlorohydroquinone (20). This reaction is catalyzed by PCP hydroxylase (28).

Given what is known about the biochemistry of halogenated phenol degradation by the *Flavobacterium* sp., two structural features of the bromoxynil molecule suggested that this molecule could be degraded by *Flavobacterium* sp. strain ATCC 39723, namely, the hydroxyl group substituted on either side with adjacent bromine atoms and the *para* cyano group. We report here on bromoxynil metabolism by the *Flavobacterium* sp., apparently catalyzed by the same enzymes which degrade PCP.

MATERIALS AND METHODS

Analytical methods and chemicals. Cell-free culture supernatants for various analyses were prepared by centrifugation of bacterial culture (12,000 × *g*, 10 min). Culture extracts for gas chromatography-mass spectrometry (GC-MS) analysis were prepared as previously described (22). Ethyl ether extracts of enzyme reaction mixtures were similarly prepared except that they were stabilized with 1 mM dithiothreitol immediately following extraction. In some cases, samples were methylated by overnight incubation with diazomethane in diethyl ether or acetylated as previously described (23).

Absorption spectra of culture supernatants were recorded with a Beckman DU7 spectrophotometer. Concentrations of aromatic compounds in aqueous samples were determined by high-pressure liquid chromatographic (HPLC) analysis as described by Xun and Orser (28) with detection by A_{284} and A_{310} or as described by Topp and Akhtar (22) with a solvent system consisting of 45% methanol–55% 5 mM NaH_2PO_4 (pH 7.0).

Cyanide concentrations were estimated by the colorimetric method of Nagashima (10) except that the assay volume was reduced proportionally to 1 ml. Cyanide standards were prepared in the incubation buffer, and sample concentrations

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were estimated by comparison with a standard curve. Preliminary experiments showed that bromoxynil did not interfere with the assay and that it was chemically stable at the pH values used in the experiments described here. Bromide ions were analyzed by the phenol red colorimetric method as described by Clesceri et al. (2) except that the assay volume was proportionally reduced to 1 ml. NADPH consumption was determined as previously described (28). Oxygen consumption was estimated by means of an oxygen electrode (Oxygraph model 5301; Yellow Springs Instrument Co., Yellow Springs, Ohio). Oxygen concentrations in the reaction mixture were calibrated by the method of Robinson and Cooper (13).

Organic chemicals of the highest purity available were purchased from Aldrich (Milwaukee, Wis.), Sigma Chemical Co. (St. Louis, Mo.), or Pfaltz and Bauer (Waterbury, Conn.) and used without further purification.

Growth and maintenance of *Flavobacterium* sp. strain ATCC 39723. Cells were grown in batch culture, and in some cases induced with PCP, in a mineral salts (MS) medium containing 4 g of L-sodium glutamate per liter as previously described (24). In some experiments, cells were grown in continuous culture under carbon limitation on 500 mg of glucose per liter as previously described (25). Cells to be used in experiments were harvested and resuspended in MS medium by the method of Topp et al. (24). Determinations of bromide production from bromoxynil were made with PCP-induced cells resuspended to an absorbance of 2.5 at 600 nm in 20 mM Tris hydrochloride buffer (pH 8.0). Coinduction experiments were done in the following fashion. Three 50-ml batch cultures of *Flavobacterium* cells in MS medium containing 10 g of sodium glutamate per liter were grown to the early log phase of growth. One culture was then supplemented with 5 mg of bromoxynil per liter, a second was supplemented with 5 mg of PCP per liter, and a third received no additions. At 1.5 h following these additions, each of the untreated, PCP-supplemented, and bromoxynil-supplemented cultures was split into two equal smaller batch cultures. One of each pair was then supplemented with 100 mg of chloramphenicol per liter to block subsequent protein synthesis. Following an additional 20-min incubation, 10-ml aliquots from each of the six batch cultures were dispensed into a series of 125-ml Erlenmeyer flasks for incubation with either PCP or bromoxynil.

Enzyme reactions with PCP hydroxylase. PCP hydroxylase was purified as described by Xun and Orser (28).

Enzyme reactions were conducted at 23°C in 100 mM potassium phosphate buffer (pH 7.0) containing 0.5% Tween 20. The enzyme concentration was 500 μg/ml in all reactions. Reaction mixtures in experiments examining the stoichiometry of bromoxynil and NADPH consumption contained 200 μM bromoxynil and 207 μM NADPH. Reaction mixtures in experiments examining the stoichiometry of oxygen consumption and cyanide production contained 100 μM bromoxynil and 400 μM NADPH.

Protein was quantified by the Bradford assay (1).

RESULTS

Degradation of bromoxynil by whole cells of *Flavobacterium* sp. strain ATCC 39723. The ability of *Flavobacterium* sp. strain ATCC 39723 to degrade bromoxynil was examined in batch cultures. Glutamate-grown cells were harvested in the mid-logarithmic phase of growth and resuspended in MS medium containing 4 g of sodium glutamate per liter and 5 mg of bromoxynil per liter. Following an overnight incubation, a strong odor of cyanide was evident in the flask headspace. The characteristic UV absorbance peak of bromoxynil at 283 nm disappeared without any accumulation of UV- or visible-light-absorbing material at other wavelengths (data not shown). HPLC analysis of spent batch culture supernatants showed the complete removal of bromoxynil with no new UV-absorbing peaks being observed. An ethyl ether extract of 3 liters of spent culture supernatant incubated as described above was prepared. A portion of the extract was acetylated. In both acetylated and unacetylated extracts, GC-MS analysis did not reveal any bromide- or cyanide-containing molecules other than residual bromoxynil (data not shown). Alternate carbon sources were not required to support bromoxynil degradation. In a 4-day incubation, 10^7 *Flavobacterium* cells per ml incubated in MS medium containing 5 mg of bromoxynil per liter as the sole carbon source completely degraded the bromoxynil as determined by HPLC. Colorimetric analysis of culture supernatants at the end of the incubation confirmed that cyanide was produced. A concentration of 0.35 mg of cyanide per liter was determined, which corresponded to 75% of the expected yield were cyanide stoichiometrically produced from bromoxynil. No cyanide was detected in cell-free controls similarly incubated, indicating that the degradation was catalyzed by the *Flavobacterium* sp.

TABLE 1. Coinduction of PCP and bromoxynil metabolism in whole cells of *Flavobacterium* sp. strain ATCC 39723

Inducer[a]	Chloramphenicol[b]	% Removal of substrate[c]	
		Bromoxynil	PCP
None	−	37	95
None	+	0	0
Bromoxynil	−	56	100
Bromoxynil	+	15	85
PCP	−	67	100
PCP	+	69	100

[a] Details concerning induction procedures are in the Materials and Methods section.
[b] 100 mg/liter.
[c] Cell suspensions (A_{600} = 0.35) were incubated with either 5 mg of bromoxynil per liter or 5 mg of PCP per liter for 1 h. Substrate concentrations were determined by HPLC at the beginning and end of the incubation period.

The metabolism of bromoxynil was accompanied by the stoichiometric accumulation of bromide. Cells incubated in the presence of 28.5 μM bromoxynil produced 56.2 μM bromide.

Bromoxynil and PCP degradation activity were coinduced (Table 1). Cells which were not preinduced with either PCP or bromoxynil degraded 37 and 95% of the 5-mg/liter bromoxynil or PCP, respectively. In the presence of chloramphenicol, however, neither of the two compounds was degraded, indicating that induction of PCP and bromoxynil degradative enzymes was required for metabolism of either of these substrates. In contrast, cells preinduced with bromoxynil degraded both this compound and PCP regardless of the presence of chloramphenicol. No UV-absorbing metabolite was detected in culture supernatants analyzed by HPLC.

Bromoxynil was degraded more slowly by the *Flavobacterium* sp. than was PCP (Table 1). One factor which could potentially contribute to the reduced rate of bromoxynil metabolism would be the accumulation of deleterious concentrations of cyanide. The *Flavobacterium* sp. was indeed sensitive to cyanide concentrations in the range produced by

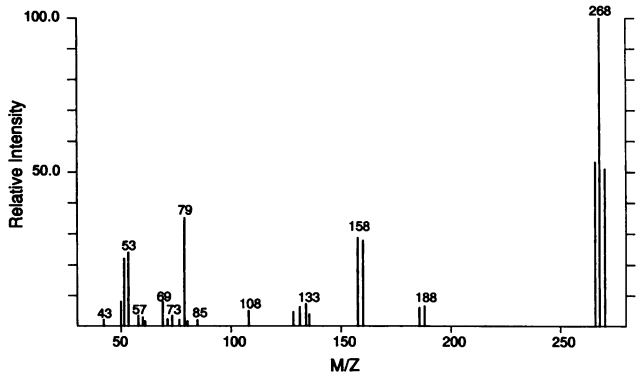

FIG. 1. Mass spectrum of the metabolite produced upon incubation of bromoxynil with PCP hydroxylase.

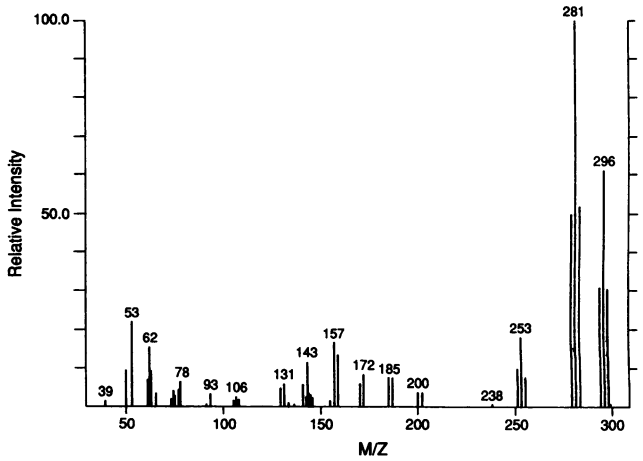

FIG. 2. Mass spectrum of the metabolite produced upon incubation of PCP hydroxylase with bromoxynil following methylation of the reaction products with diazomethane.

bromoxynil metabolism. For example, a cyanide concentration of 0.9 mg/liter, corresponding to that which would be produced from 10 mg of bromoxynil per liter, inhibited the growth of the *Flavobacterium* sp. in MS medium containing 1 g of glucose per liter by 92% compared with controls not receiving cyanide (data not shown).

Bromoxynil metabolism by purified PCP hydroxylase. Enzyme reaction mixtures completely degraded 100 μM bromoxynil within 20 min. HPLC analysis of the reaction mixture at the end of the incubation revealed the disappearance of bromoxynil (retention time of 9.4 min, HPLC method of Xun and Orser [28]) and the accumulation of an end product with a retention time of 7.9 min. GC-MS analysis of the culture supernatant revealed a compound (retention time, 5.5 min) with a mass spectrum consistent with that expected for dibromohydroquinone (DBHQ) (Fig. 1). The relative intensity and molecular weight of the peaks at *m/z* 270 (M + 4), 268 (M + 2), and 266 (M^+) are consistent with a molecular formula of $C_6Br_2H_4O_2$ given the natural abundance of 50.7 and 49.3% for ^{79}Br and ^{81}Br, respectively. Major fragments are observed at *m/z* 188 (loss of $H^{79}Br$ from 268; loss of $H^{81}Br$ from 270), 186 (loss of $H^{79}Br$ from 266; loss of $H^{81}Br$ from 278), 160 (loss of CO from 188), and 156 (loss of CO from 186). Methylation of the culture extract with diazomethane yielded a compound with a retention time of 5.16 min and a mass spectrum consistent with that expected for dibromodimethoxybenzene with a molecular formula of $C_8Br_2H_8O_2$ (Fig. 2). The characteristic two-bromine pattern of the parent molecule is evident with peaks at *m/z* 294 (M^+), 296 (M + 2), and 298 (M + 4). Major fragments are observed at *m/z* 279, 281, and 283 (loss of CH_3 from 294, 296, and 298, respectively); 251, 253, and 255 (loss of CO from 279, 281, and 283, respectively); 202 (loss of ^{79}Br from 281; loss of ^{81}Br from 283); 200 (loss of ^{79}Br from 279; loss of ^{81}Br from 281); and 187 and 185 (loss of CH_3 from 202 and 200), respectively.

Quantitative determinations of substrate consumption and cyanide production revealed that bromoxynil, oxygen, and NADPH were consumed in a 1:1:2 ratio and that 1 mol of cyanide was produced per mol of bromoxynil consumed (data not shown). Bromine was not detected in enzyme reaction mixtures. The concentration of the accumulated postulated DBHQ could not be determined in the absence of authentic standards.

In contrast to the results obtained with whole cells, PCP hydroxylase catalyzed the degradation of bromoxynil much more rapidly than it did that of PCP. The specific activities of reaction mixtures containing 100 μM haloaromatic substrate and 400 μM NADPH were 50.8 and 8.6 μmol/min/mg of protein for bromoxynil and PCP, respectively.

DISCUSSION

Our overall results indicate that *Flavobacterium* sp. strain ATCC 39723 degraded bromoxynil with the same enzymes as those that are used to degrade PCP. Bromoxynil-degrading activity was coinduced with PCP-degrading activity, either substrate serving as an inducer. PCP was a better inducer than bromoxynil. Whole cells degraded bromoxynil without the accumulation of any intermediate detectable by UV spectroscopy, HPLC, or GC-MS, indicating ring opening of the aromatic nucleus. Bromide and cyanide were products of bromoxynil degradation by whole cells. Two moles of bromide were produced per mole of bromoxynil consumed, indicating complete dehalogenation of the molecule. Cyanide production by purified PCP hydroxylase, but not whole cells, was stoichiometric. Since the *Flavobacterium* sp. does not metabolize cyanide when cyanide is supplied to growing cultures as the sole nitrogen source (data not shown), we speculate that volatilization, complexation, or adsorption is a mechanism which may underlie the 25% lower recovery of cyanide from what was expected. In addition to cyanide, purified PCP hydroxylase converted bromoxynil to an end product which was more hydrophilic than PCP when analyzed by HPLC and whose mass spectrum strongly suggested it was DBHQ (Fig. 1 and 2).

These results are consistent with the pathway of bromoxynil metabolism proposed in Fig. 3. Metabolism is initiated by the action of PCP hydroxylase, which replaces the cyano substituent in the 4 position with a hydroxyl group, yielding

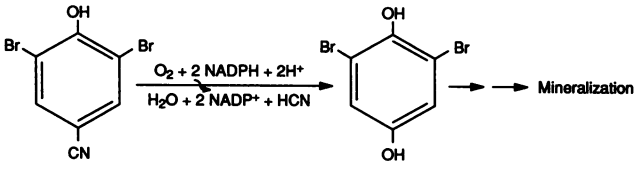

FIG. 3. Proposed pathway for bromoxynil metabolism by *Flavobacterium* sp. strain ATCC 39723.

2,6-DBHQ. The intermediate 2,6-DBHQ is then proposed to be mineralized by as yet unspecified reactions catalyzed by a suite of enzymes coinduced by PCP or bromoxynil. This pathway is analogous to that proposed by Steiert and Crawford (20) for the degradation of PCP by *Flavobacterium* sp. strain ATCC 39723 with the following three provisos. First, reductive dehalogenation reactions proposed by Steiert and Crawford (20) to account for the conversion of tetrachloro-*p*-hydroquinone to 2,6-dichlorohydroquinone are not required for the degradation of bromoxynil. However, this enzymatic activity is expressed in bromoxynil-induced cells since they also degrade PCP without the accumulation of detectable intermediates. Second, 2,6-DBHQ is metabolized by the same reactions as 2,6-dichlorohydroquinone. This proposal is consistent with the observation that 2,4,6-tribromophenol is mineralized by the *Flavobacterium* sp. (27). Finally, the PCP hydroxylase catalyzing the initial attack at the 4 position can also replace a cyano substituent with a hydroxyl group.

The observation that PCP hydroxylase converts bromoxynil to DBHQ without the production of bromide or any other detectable metabolite suggests that this enzyme is restricted in its activity to an attack on the *para* position of halogenated phenols.

This observation extends the range of reactions catalyzed by the enzyme to cyanogenesis. It was previously observed that PCP degradation catalyzed by PCP hydroxylase was accompanied by the consumption of 2 mol of NADPH per mol of chloride produced from PCP (28). The same stoichiometry accompanying the production of cyanide from bromoxynil suggests that the enzymatic mechanisms of dehalogenation and cyanogenesis are similar.

Although bromoxynil is degraded much more slowly by whole cells than is PCP, the rate of bromoxynil turnover by PCP hydroxylase is six times that of PCP. This may be because PCP enters whole cells more rapidly than does bromoxynil. Alternatively, the toxicity of the cyanide produced upon bromoxynil metabolism may very well inhibit the activity of whole cells. There was no indication of cyanide inhibition of PCP hydroxylase since the reaction was linear throughout the assay (data not shown).

The cyano group is a good potential leaving group. It is about the same size as chlorine or bromine and is more electron withdrawing than chlorine, with a strong dipole moment with the negative end oriented towards the nitrogen atom (15).

This is the first report of metabolism of a benzonitrile derivative accompanied by cyanogenesis. Renganathan and Johnston (12) reported the fortuitous production of small amounts of cyanide from benzonitrile upon its cometabolism by enzymes of the toluene pathway in *Pseudomonas* sp. strain T-12. These researchers postulated that acyl cyanide was displaced from the benzonitrile ring-cleavage product by catechol 2,3-dioxygenase, resulting in suicide inactivation in a manner analogous to that observed with 3-halocatechols.

Bromoxynil degradation in soil, in plants, and in mammals has hitherto been considered to occur via hydrolysis of the cyano group, or via ring hydroxylation at unsubstituted positions followed by conjugation (5). We do not know whether the reaction catalyzed by the PCP hydroxylase occurs in other systems or whether cyanogenesis from benzonitrile derivatives has any significance in nature. However, pyrolysis soft-ionization MS analysis of soil humic material indicates that it contains benzonitrile derivatives (14), and reactions removing cyanide from aromatic nuclei might therefore contribute to the production of cyanide in soil.

Previous bromoxynil-degrading bacteria examined metabolized the molecule by hydrolyzing the cyano substituent to the carboxylate. For example, a *K. pneumoniae* subsp. *ozaenae* strain used bromoxynil as a nitrogen but not a carbon source, accumulating 3,5-dibromo-4-hydroxybenzoate as the end product of metabolism (7). The gene encoding the bromoxynil-degrading nitrilase has been used to generate bromoxynil-resistant transgenic plants (19). The rate of bromoxynil catalysis by PCP hydroxylase is more rapid than that effected by the *K. pneumoniae* nitrilase. Although we have not determined the kinetic properties of bromoxynil hydroxylation by PCP hydroxylase, the specific activity determined in our standard assay of 50.8 μmol/min/mg of protein is greater than the V_{max} of 15.0 μmol of ammonia released per min per mg of protein reported for purified nitrilase (18).

The pathway of bromoxynil metabolism described in our report is a novel one resulting in the mineralization of this herbicide. We suggest that in addition to its utility for decontaminating soils and water polluted with PCP, the *Flavobacterium* sp. may be useful for decontaminating bromoxynil spills and containers in cases in which the herbicide concentration is low enough to avoid cyanide toxicity.

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