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Role of electron-donating cosubstrates in the anaerobic biotransformation of chlorophenoxyacetates to chlorophenols by a bacterial consortium enriched on phenoxyacetate

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Abstract

A bacterial consortium that anaerobically mineralized phenoxyacetate, with transient production of phenol as an intermediate, was obtained from a methanogenic aquifer site near the Norman, OK municipal landfill. This consortium was able to convert the eight halogenated chlorophenoxyacetates tested to the corresponding chlorophenols. The chlorophenols were not subsequently metabolized. The addition of reduced substrates increased the rate of degradation of all chlorophenoxyacetates, with 78% of mono- and di-chlorinated substrates being transformed to chlorophenols in butyrate-amended cultures, compared to less than 37% transformed in unsupplemented cultures. Butyrate increased the transformation of 2,4,5-trichlorophenoxyacetate from 10% to 20%. An experiment evaluating the effects of several compounds on the side-chain cleavage reaction of 3-chlorophenoxyacetate showed that addition of compounds which readily act as hydrogen donors (butyrate, crotonate, ethanol, propionate, and hydrogen) resulted in 2 to 5 times the amount of 3-chlorophenoxyacetate transformed compared to controls with no amendment, formate had a slight stimulatory effect, and acetate and methanol had no effect. Butyrate addition also increased the rate of phenoxyacetate degradation, resulting in transient phenol accumulation not observed in butyrate-unamended controls. These results support the hypothesis that the side-chain cleavage of phenoxyacetate is a reductive process that is stimulated by the oxidation of reduced cosubstrates.

Introduction

Two pathways for the anaerobic biodegradation of the herbicide 2,4,5-trichlorophenoxyacetate (2,4,5-T) have been noted, in which aryl dehalogenation (DeWeerd et al. 1986; Gibson & Suflita 1986, 1990; Suflita et al. 1984) or cleavage of the side-chain (Mikesell & Boyd 1985; Eder 1980) occur as the initial metabolic transformation. Even when reductive dehalogenation is the primary biotransformation route, evidence suggests that side-chain cleavage occurs before the aromatic nucleus is mineralized (Gibson & Suflita 1990). This results in the transient appearance of phenol and a variety of di- and monochlorophenols during anaerobic 2,4,5-T metabolism, in addition to several chlorophenoxyacetates (Gibson & Suflita 1986, 1990). In order to study the side-chain cleavage reaction without the complicating dehalogenation reactions, we enriched a methanogenic bacterial consortium for the

ability to completely mineralize phenoxyacetate. This enrichment produced phenol and chlorophenols as intermediates of phenoxyacetate and chlorophenoxyacetate decomposition, respectively. The chlorophenols were not mineralized by the consortium. We were able to use this enrichment to probe the environmental factors influencing the removal of the side-chain of phenoxyacetate herbicides and related intermediates.

Materials and methods

Enrichment of the consortium

The enrichment was obtained from phenoxyacetate-amended slurries made with aquifer solids and ground water from a methanogenic aquifer site located near the landfill in Norman, OK (Beeman & Suflita 1987). The slurries were made with 100g (wet weight) aquifer sediments and 25ml of anaerobic ground water, as previously described (Gibson & Suflita 1986). Transfers (~10%) were made from slurries in which phenoxyacetate had been degraded to the anaerobic basal salts medium of Shelton and Tiedje (1984), except that the $NaHCO_3$ concentration was increased to 3g/l, and $CoCl_2 \cdot 6H_2O$ and $NiCl_2 \cdot 6H_2O$ were reduced to 0.03mg/l. The medium also had a 1mM starting concentration of sodium sulfide. The aquifer slurry and subsequent transfers were amended with 0.5 to 1mM phenoxyacetate as a carbon and energy source.

Experimental protocol

An enriched phenoxyacetate-degrading bacterial consortium maintained for over 18 months on phenoxyacetate was used for all experiments. Subcultures (10 or 25ml) were aseptically dispensed from a larger (2-1) batch culture into aluminum seal tubes (Bellco Glass, Inc., Vineland, NJ) or 50ml serum bottles while the containers were flushed with an oxygen-free gas mixture (80% N_2/20% CO_2). The bottles were sealed with sterile 1cm black rubber septa (Bellco Glass, Inc.) and aluminum crimp seals. The headspace of the bottles was exchanged three times by evacuating and then refilling with the O_2-free gas mixture and over-pressurized to 140kPa. To determine the effects of hydrogen on phenoxyacetate degradation by the culture, the headspace was adjusted to 80% H_2/20% CO_2 and overpressurized to 34 or 140kPa. The headspace of these cultures was replenished with the gas mixture when the headspace pressure decreased to atmospheric pressure or below. Stock solutions of test substrates were filter-sterilized and added to the culture to result in an initial concentration of 500–750μM for chlorophenoxyacetates and 1mM for fatty acids and alcohols. Treatments were performed in triplicate, and an autoclaved sample served as a sterile control. The cultures were incubated at room temperature, in the dark. Bottles containing a hydrogen headspace were shaken at 250rpm, while all other experiments were incubated without agitation. Subsamples were periodically withdrawn from the experiments and stored at −10° C.

Analysis

Subsamples were thawed and centrifuged at 20,000× g for 25 minutes prior to HPLC analysis of the supernatant. Chlorophenoxyacetates and chlorophenols were analyzed by C18 reverse-phase chromatography as previously described (Gibson & Suflita 1986). Fatty acid analysis was also by HPLC but an Aminex HPX-87X ion exclusion column, 300× 7.8mm (Bio-Rad, Richmond, CA) was used. The mobile phase was 0.016N sulfuric acid and the flow rate was 0.9ml/min. Detection was by UV absorbance at 210nm.

Chemicals

Most chemicals were commercially available and used without further purification. The 2,5-dichlorophenoxyacetate and the 3-chlorophenoxyacetate were synthesized by the method of Koelsch (1931) with the addition of a final benzene wash to remove any unreacted chlorophenol.

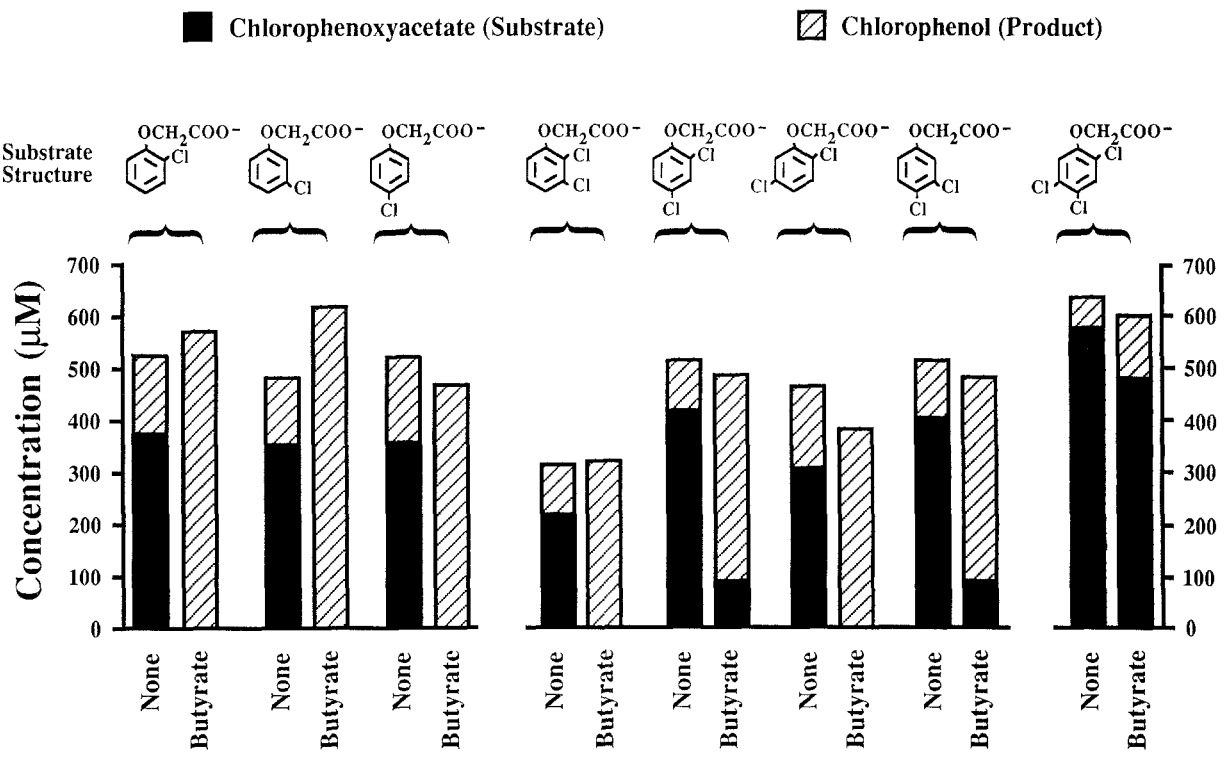

Fig. 1. Biotransformation of chlorophenoxyacetates by the phenoxyacetate-degrading consortium in the presence and absence of butyrate. Incubation time was 13 days and no substrate depletion was noted in autoclaved controls.

Results

In initial experiments with the enrichment, phenoxyacetate depletion was measured, and phenol transiently accumulated. However, after two transfers, phenoxyacetate degradation did not begin. We theorized that the loss of the metabolic activity might be due to the depletion of a required cosubstrate. In an attempt to recover phenoxyacetate-degrading activity in the enrichment, we added either short-chain acids or an alcohol (1mM) to several transfers. The butyrate- and propionate-amended cultures regained the ability to degrade phenoxyacetate, while cultures amended with acetate or methanol did not. These former two enrichments were subsequently maintained with phenoxyacetate as the sole carbon source for over 18 months.

We tested the enrichments for the ability to biodegrade the three mono-, four dichloro-, and one trichloro-phenoxyacetate congeners. The transformation of each chlorophenoxyacetate was tested in both the presence and absence of either butyrate or propionate. Because the results were similar with both enrichments, only results obtained with the butyrate-stimulated enrichment are reported, and that enrichment was used for all subsequent experiments.

All chlorophenoxyacetates examined were transformed stoichiometrically to the corresponding chlorophenols by the consortium (Fig. 1). No aromatic intermediates other than the chlorophenols were observed. The chlorophenol products persisted, and there was no evidence for any further biodegradation of these metabolites. Greater biotransformation of the chlorophenoxyacetates was measured in cultures treated with butyrate. At least 78% of the test mono- and di-chlorophenoxyacetates were transformed in butyrate-amended cultures, while less than 37% of the parent substrates were degraded in unsupplemented cultures. The biotransformation of 2,4,5-T was only slightly

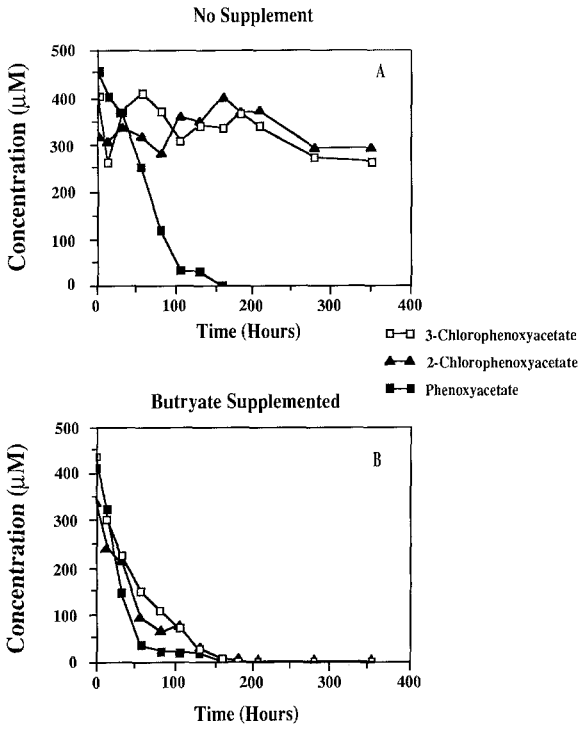

Fig. 2. Degradation of phenoxyacetate, 2-chlorophenoxyacetate, and 3-chlorophenoxyacetate with no additional organic supplement (A) and with 1 mM butyrate added (B).

stimulated in the presence of butyrate (Fig. 1), and no increase in substrate removal was noted with further incubation.

An experiment was designed to monitor the rate of phenoxyacetate, 2-chloro-, or 3-chlorophenoxyacetate disappearance in the presence and absence of butyrate (Fig. 2). The concentration of 2-chlorophenoxyacetate and 3-chlorophenoxyacetate declined very slowly ($\sim 0.3\,\mu M \cdot h^{-1}$) in unsupplemented samples, while phenoxyacetate was degraded at about $5\,\mu M \cdot h^{-1}$. However, when butyrate was added, all three compounds declined at comparably rapid rates (Fig. 2).

The effect of butyrate on the disappearance of phenoxyacetate and the appearance of the phenol intermediate is shown in Fig. 3. Phenoxyacetate degradation was almost twice as fast in the presence of butyrate (5 vs. $\sim 10\,\mu M \cdot h^{-1}$), and phenol accumulated transiently under these conditions. In the absence of butyrate, phenoxyacetate metabolism was slower, and phenol did not accumulate.

In order to compare the effect of butyrate and propionate on the side-chain cleavage reaction, the enrichment was amended with 3-chlorophenoxyacetate and one of these supplements. The halogenated substrate was used to eliminate potential effects from ring-cleavage intermediates. Both supplements increased the biotransformation of 3-chlorophenoxyacetate by the consortium, but butyrate exhibited the greater effect (Fig. 4). Butyrate supplementation resulted in a maximum biotransformation rate of about $8\,\mu M \cdot h^{-1}$, while propionate supplementation resulted in a rate of $1\,\mu M \cdot h^{-1}$. Butyrate degradation by the enrichment began immediately and was complete within 80 hours (Fig. 4). Propionate metabolism was slower than butyrate degradation, with more than 70% of the added propionate still present at the end of the experiment (data not shown). The stimulatory effect of propionate on 3-chlorophenoxyacetate metabolism over the unsupplemented control was not evident until after 104 h of incubation (Fig. 4).

In order to confirm the effect of other short-chain organic acids and alcohols on the side-chain cleavage reaction, subcultures were amended with 3-chlorophenoxyacetate and with various supplements. After 12 days, propionate, butyrate, crotonate, and ethanol additions stimulated the bioconversion of 3-chlorophenoxyacetate (Table 1). Cultures supplemented with these compounds transformed about two to four times as much substrate as the unsupplemented controls. Degradation of the chlorophenoxyacetate in the presence of acetate or methanol supplements was not different from unsupplemented controls. Formate addition resulted in 42% more substrate transformed than nonsupplemented cultures. Hydrogen addition also stimulated the side-chain cleavage reaction relative to unsupplemented controls, but no significant difference between the cultures overpressurized by 34 kPa or 140 kPa was detected.

Discussion

In the enrichment, phenoxyacetate was transformed to phenol, which was subsequently degraded. Similarly, all of the chlorinated phenoxyace-

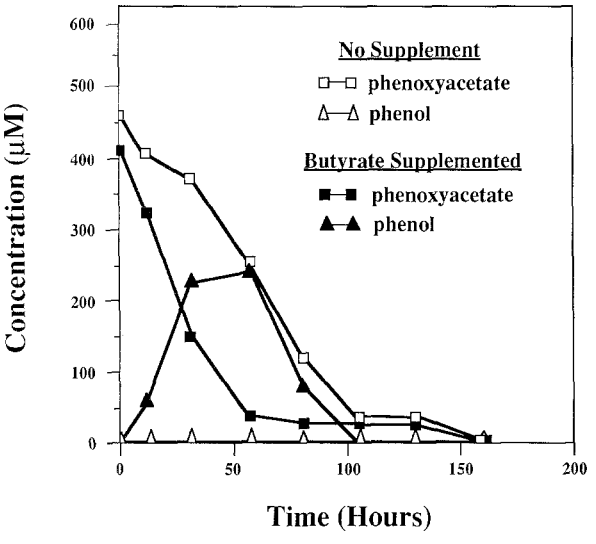

Fig. 3. Biodegradation of phenoxyacetate and the production of phenol in the presence and absence of supplemental butyrate.

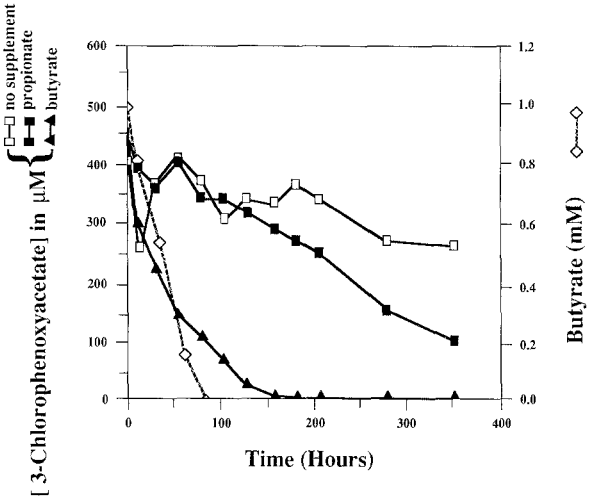

Fig. 4. Biodegradation of 3-chlorophenoxyacetate with no supplement, 1 mM butyrate, or 1 mM propionate. The degradation of butyrate is also shown.

tates examined were transformed by the consortium to the corresponding chlorophenolic derivatives, but these products were not degraded further. Unlike the other halophenoxyacetates, only a small amount of 2,4,5-trichlorophenol was formed from 2,4,5-T metabolism by the consortium (Fig. 1). The decreased transformation of this substrate relative to the other chlorophenoxyacetates may be due to the steric hindrance associated with the trihalogenated compound or to the toxicity of the accumulated 2,4,5-trichlorophenol.

The slower degradation of the test monochlorinated phenoxyacetates relative to phenoxyacetate itself was overcome when butyrate was added to the consortium. This result indicated that the absence of a second substrate, rather than the presence of an aryl halide, limited the degradation of the chlorophenoxyacetates. This second substrate apparently is provided during the degradation of the aromatic nucleus of phenoxyacetate by the consortium when the unchlorinated substrate is the sole organic carbon and energy source. If phenol degradation intermediates are required for the initial bioconversion of phenoxyacetate by this consortium, then phenoxyacetate catabolism should be limited by the rate of phenol degradation.

When phenoxyacetate was the sole organic carbon source for the consortium, phenol was used as quickly as it was produced and did not accumulate above the detection limit of 10μM. If, as hypothesized, the initial conversion of phenoxyacetate is a reductive process with the rate dependent on the release of reducing equivalents for aromatic ring metabolism, then addition of another oxidizable carbon source might increase the rate of phenoxyacetate metabolism. When both butyrate and phenoxyacetate were added to the consortium, the rate of phenoxyacetate metabolism exceeded the rate

Table I. Effects of organic supplements and H_2 on the conversion of 3 chlorophenoxyacetate to 3-chlorophenol.

Treatment[a]	3-chlorophenoxyacetate degraded[b] (μM± standard deviation)
None	121.4± 8.9
34 kPa H_2/CO_2	232.0± 43.6
140 kPa H_2/CO_2	257.6± 16.7
Formate	174.2± 13.4
Methanol	115.3± 43.5
Acetate	111.0± 19.1
Ethanol	293.2± 7.3
Propionate	255.9± 11.2
Butyrate	512.6± 36.1
Crotonate	399.0± 36.9

[a] 1 mM organic supplements.

[b] Initial concentration of 3-chlorophenoxyacetate was 750μM, incubation time was 12 days.

of phenol degradation. This lends support to the hypothesis that the rate of the initial conversion of phenoxyacetate is at least partially controlled by the rate of phenol metabolism in the absence of other sources of reducing equivalents.

A small amount of chlorophenoxyacetate metabolism was observed even in the absence of an added electron donor (Figs 1 and 2). This may be due to the carry over of oxidizable compounds from the parent consortium, the presence of reduced components in the cells themselves, or some release of reducing equivalents from the metabolism of the side-chain of the chlorophenoxyacetates.

Table 1 shows that not all supplements were effective in stimulating the conversion of 3-chlorophenoxyacetate to a chlorophenol. Those organic compounds that are good hydrogen donors (butyrate, propionate, ethanol, crotonate, and formate) stimulated the side-chain cleavage reaction. Similarly, hydrogen supplementation resulted in more biotransformation of the chlorophenoxyacetate than in the unsupplemented controls. It is unclear why cultures pressurized with H_2/CO_2 to 34 kPa transformed essentially the same amount of 3-chlorophenoxyacetate as those pressurized to 140 kPa. However, when the pH was examined after 10 days incubation, the culture fluid decreased from its initial value of 6.8 to <6 in incubations pressurized to 140 kPa with the H_2/CO_2 mixture, while it was still 6.4 in experiments pressurized to 34 kPa. This indicates that acetogenic processes may have also been occurring in the enrichments. It is possible that acetogenesis could act as an alternate hydrogen sink. Also, the lower pH may have inhibited the bacteria responsible for the side-chain transformation.

Based on the observed stimulation when reduced substrates are added, the first step in anaerobic phenoxyacetate metabolism appears to be a reduction requiring the concurrent oxidation of another substrate. The proposed reaction is:

$$C_6H_5OCH_2COO^- + 2e^- \; 2H^+$$
$$4\ C_6H_5OH + CH_3COO^-$$

In this respect, it is interesting to note the work of Krumholz and Bryant (1986). They isolated an acetogenic bacterium that required sugars as electron donors for growth on methoxylated aromatic compounds. It may be that the electron donor was needed to reduce the ether bond of methoxylated compounds in a manner consistent with our observations on phenoxyacetate metabolism. This is in contrast to the alkyl ether cleavages reported by several groups (Dwyer & Tiedje 1983, 1986; Schink & Stieb 1983; Straß & Schink 1986; Tanaka & Pfennig 1988; Wagener & Schink 1988) for which a hydrolytic reaction is likely, and the aryl ether cleavages carried out by acetogens that do not require a supplemental electron donor for O-demethylation of and growth on methoxylated aromatic compounds (Bache & Pfennig 1981; Mountfort & Asher 1986). Because reductive processes would be stimulated by a suitable electron donor whereas hydrolytic processes are probably not, it may be that the aryl ether bond cleavage observed during phenoxyacetate degradation is an example of the former process.

The mechanism(s) of anaerobic ether cleavage reactions are of great importance. This reaction may be involved in the anaerobic metabolism of many compounds including phenoxy acid and phenyl ether herbicides, various lignin components, and ether linked polymers such as those found in nonionic surfactants. Optimization of the ether cleavage reaction may facilitate anaerobic removal of pollutants which contain ether bonds.

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