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A J Weightman, A L Weightman and J H Slater
Appl. Environ. Microbiol. **1985, 49(6):1494.**

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1985, p. 1494–1501
0099-2240/85/061494-08$02.00/0

Vol. 49, No. 6

Toxic Effects of Chlorinated and Brominated Alkanoic Acids on *Pseudomonas putida* PP3: Selection at High Frequencies of Mutations in Genes Encoding Dehalogenases

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Received 13 November 1984/Accepted 26 February 1985

Mutant strains of *Pseudomonas putida* PP3 capable of utilizing monochloroacetate (MCA) and dichloroacetate (DCA) as the sole sources of carbon and energy were isolated from chemostat cultures. The mutants differed from the parent strain in that they could grow on products of MCA and DCA dehalogenation (catalyzed by inducible dehalogenases I and II) and were resistant to growth inhibition by the two substrates. The growth inhibition of strain PP3 by MCA, DCA, and other halogenated alkanoic acids was studied. Sensitivity to dehalogenase substrates was related to the expression of the dehalogenase genes. For example, mutants producing elevated levels of one or both of the dehalogenases were sensitive to 2-monochloropropionate and 2-monochlorobutanoate at concentrations which did not affect the growth of strain PP3. *P. putida* PP1, the parent of strain PP3, was resistant to the inhibitory effects of MCA and DCA. Spontaneous mutants of strain PP3, also resistant to MCA and DCA, were selected at high frequency, and four different classes of these strains were distinguished on the basis of dehalogenase phenotype. All dehalogenase-producing mutants were inducible; no constitutive mutant has yet been isolated. Most of the resistant mutants examined did not produce one or both of the dehalogenases, and over half of those tested failed to revert back to the parental (strain PP3) phenotype, indicating that the observed mutations involved high-frequency deletion of DNA base sequences affecting expression of genes encoding dehalogenases and associated permease(s).

Pseudomonas putida PP3 can utilize 2-monochloropropionate (2MCPA), 2-monobromopropionate (2MBPA), and 2,2-dichloropropionate (22DCPA) as sole carbon and energy sources as a result of the induction of two dehalogenase enzymes by these substrates (20, 22). These enzymes differ from each other with regard to their substrate specificities, molecular weights, and other physical properties (22, 23) but are coinduced by a variety of halogenated compounds (20; A. J. Weightman, Ph.D. thesis, University of Warwick, England, 1981). Monochloroacetate (MCA), dichloroacetate (DCA), and 2-monochlorobutanoate (2MCBA) are all inducing substrates of dehalogenases I and II. Dehalogenase activity against MCA in fully induced cell extracts of strain PP3 is divided almost equally between the two dehalogenases, whereas 90% of activity with DCA is associated with dehalogenase I, and 2MCBA activity is associated almost entirely with dehalogenase II (22; Weightman, Ph.D. thesis). MCA, DCA, and 2MCBA, despite inducing dehalogenase levels commensurate with good growth on these substrates, cannot be utilized as sole carbon and energy sources by strain PP3 (20, 21). Both dehalogenases catalyze the hydrolytic cleavage of 2-haloalkanoates and 2,2-dihaloalkanoates to form 2-hydroxyalkanoates and 2-ketoalkanoates, respectively. The dehalogenated products (lactate and pyruvate, respectively, in the cases of 2MCPA and 22DCPA) can be channeled into central metabolism. Growth on halogenated acetates has been described for several different soil microorganisms (7, 9, 11, 15), although toxicity of substrates other than monofluoroacetate to dehalogenase-producing organisms was not reported in these papers. A *Rhizobium* species isolated by Berry et al. (3) produced high levels of dehalogenase activity against MCA in cell extracts after induction with 22DCPA, but MCA was not a growth substrate for the organism, presumably because it did not induce the dehalogenases.

To date, no study has considered the genetics of dehalogenase expression in *P. putida* PP3 or the other dehalogenase-producing organisms described by Goldman and his colleagues (7–9) and by Little and Williams (15). Kawasaki et al. isolated a monofluoroacetate-utilizing strain of *Moraxella* sp. containing two dehalogenases, both of which were reported to be plasmid encoded (11, 13). The two enzymes are apparently coded by genes which are under separate regulatory control. One is inducible by monofluoroacetate, and the other is constitutive; the constitutive enzyme was purified (12). Good evidence for the presence of large (100- to 200-megadalton) plasmids encoding dehalogenases in a variety of soil microorganisms has been obtained in our laboratory (D. J. Hardman, P. C. Gowland, and J. H. Slater, Appl. Environ. Microbiol., in press).

Substrate toxicity has been recognized as one barrier to the utilization of some dehalogenase substrates as growth substrates by *P. putida* PP3 (20, 21). The aim of the present study was to examine the relationship between the toxicity of some haloalkanoates and dehalogenase gene expression and to characterize mutant strains derived from strain PP3 which differ phenotypically in regulation or expression, or both, of genes encoding dehalogenases and associated functions.

MATERIALS AND METHODS

Isolation, maintenance, and growth of bacteria. *P. putida* strains PP1 and PP3 were isolated from a microbial community grown on the herbicide 2,2-dichloropropionate

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(22DCPA) (19). Parent and mutant strains were grown in defined medium consisting of a basic mineral salts medium previously described (19) supplemented with a carbon source as appropriate. Strain PP309, a mutant of PP3 which synthesizes elevated levels of dehalogenase I, was isolated from a chemostat culture after 6 months of continuous-flow culture on 2MCPA and 2MCBA as growth-limiting carbon sources (21). Strain PP3(R68.44) was a transconjugant isolated from a membrane mating between auxotrophic donor *Pseudomonas aeruginosa* PAC174(R68.44) and the recipient *P. putida* PP3. It was maintained after purification in a 2MCPA-limited chemostat culture. The chromosome-mobilizing ability of the plasmid was rapidly lost in this system, although the plasmid was maintained in the population throughout the period of continuous-flow culture (Weightman, Ph.D. thesis).

Mutant strains of *P. putida* resistant to growth inhibition by chlorinated acetates were isolated from overnight closed cultures of *P. putida* PP3 grown on either succinate or 2MCPA which were plated at appropriate dilutions onto solid medium containing succinate (0.5 g of carbon liter^{-1}, 10.4 mM) and either DCA or MCA (1.0 to 2.0 g of carbon liter^{-1}, 42 to 83 mM). The mutants obtained were maintained on succinate-defined medium (0.5 g of carbon liter^{-1}) after purification.

The method of Ornston et al. (16) was used to obtain mutant strains of *P. putida* PP3 unable to grow on 2MCPA. Mutants were enriched by growing *P. putida* PP3 overnight in 10 ml of defined medium with lactate (0.5 g of carbon liter^{-1}) as the sole carbon and energy source, harvesting the cells and washing them aseptically in 10 ml of sterile basic mineral salts medium, and then inoculating the cell suspension into 10 ml of defined medium containing 2MCPA (0.5 g of carbon liter^{-1}) as the sole carbon and energy source. The culture was incubated with shaking for 2 h at 30°C (until the onset of growth), and then the antibiotics cycloserine and penicillin G were added to final concentrations of 0.1 mg ml^{-1} and 10,000 U ml^{-1}, respectively. The culture was incubated with shaking at 30°C for another 5 h, during which time lysis of growing organisms was observed. The culture was then harvested and washed in 10 ml of basic mineral salts medium, suspended in sterile glass-distilled water, and shaken vigorously for 20 min to effect further lysis. To complete the cycle, 0.1 ml of the lysed cell suspension was inoculated into 10 ml of lactate-defined medium to grow overnight. After three cycles, the culture was spread plated at an appropriate dilution onto solid defined medium containing both succinate (0.1 g of carbon liter^{-1}) and 2MCPA (0.5 g of carbon liter^{-1}) and was incubated for 48 h at 30°C. Mutants unable to grow on 2MCPA produced very small colonies on this medium and were easily distinguishable from strains utilizing both carbon sources.

Continuous-flow cultures were maintained in chemostats by previously described procedures (21; Weightman, Ph.D. thesis). Samples were removed at regular intervals from the culture vessel via a sampling port and were used to estimate culture biomass, viable organisms, and substrate concentrations.

Strains were maintained and grown as previously described (20, 21) and were checked regularly for appropriate phenotypic markers. Strain purity was checked by plating stock and liquid cultures onto King medium B (14).

Measurement of dehalogenase activity in cell extracts. Dehalogenase activity was measured as previously described (21; Weightman, Ph.D. thesis). One unit of dehalogenase activity was defined as the amount of enzyme catalyzing the conversion of 1 μmol of MCA min^{-1}. The principle of the assay involved measuring free chloride ions released during the assay period by using a Marius Chlor-O-Counter (Labo International, Delft, The Netherlands).

A qualitative and rapid microassay for dehalogenase activity was performed as follows. Two or three colonies of the organism under investigation were removed from the surface of an agar plate after 24 to 48 h of incubation and suspended in 50 μl of 20 mM Tris-sulfate buffer (pH 7.9). The cell suspension was added to 55 μl of 0.6 M Tris-sulfate buffer (pH 7.9) and 91 μl of glass-distilled water in one compartment of a microtiter assay tray. The enzyme reaction was initiated by the addition of 50 mM MCA (4.7 μl of 20% [wt/vol] MCA), and the microtiter assay tray was incubated at 30°C for 30 min. Dehalogenase activity in the strains tested was estimated qualitatively by the addition of 10 μl of 0.1 M $AgNO_3$ to each compartment to precipitate as insoluble AgCl any chloride released during the incubation. The precipitate darkened considerably within 10 to 15 min when exposed to UV light, and positive reactions were scored by comparisons with appropriate control assays.

Polyacrylamide gel electrophoresis. The two dehalogenases in *P. putida* strains were separated by discontinuous polyacrylamide gel electrophoresis of cell extracts and were stained as previously described (10, 21).

A rapid screening technique for detection of dehalogenases in small volumes of induced cultures was developed. A sample (4 to 5 ml) from an overnight culture was harvested in an Eppendorf centrifuge tube, and the pellet was washed once in ice-cold 20 mM Tris-sulfate buffer (pH 7.0) and resuspended in 100 μl of the same buffer. Ice-cold lysozyme (25 μl at a concentration of 5 mg ml^{-1}, freshly prepared) and EDTA (75 μl at a concentration of 0.25 M [pH 8.0]) were added, and the suspension was kept on ice for 30 min with occasional mixing. Before electrophoresis, 10 μg of 0.05% (wt/vol) bromophenol blue was added to the mixture, and samples of 20 to 50 μl were loaded onto the gel. The remaining procedures were as previously described (21).

Oxygen-electrode experiments. Respiration by cell suspensions in the presence of various carbon sources was estimated by oxygen uptake determined by an oxygen-electrode cell (Rank Brothers, Bottishman, Cambridge, England) with a 3-ml working volume and a constant temperature of 30°C regulated by a Churchill circulator (Scientific Industries International, Inc., Loughborough, Leicester, England). Oxygen consumption was monitored with a Kipp and Zonen (Delft, The Netherlands) chart recorder which was calibrated between 100% oxygen saturation (air-saturated 20 mM NaH_2PO_4-Na_2HPO_4 buffer) and 0% oxygen saturation (oxygen removed by the addition of sodium dithionite). Washed cell suspensions in 20 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 7.0) were incubated in the electrode cell in the absence of substrate for 5 to 10 min, during which time the endogenous respiration rate was measured. The carbon sources were added to cell suspensions at concentrations ranging between 0.01 and 10 mM, and after equilibration, the rate of respiration was measured.

Chemicals. All chemicals used were of the highest purity commercially available, and the purity of alkanoic acids was checked by ^{13}C nuclear magnetic resonance when necessary (Weightman, Ph.D. thesis).

RESULTS

Growth inhibition by halogenated alkanoic acids. Growth of *P. putida* PP3 was markedly inhibited by the addition of either MCA or DCA (20 mM) to closed cultures growing on

succinate during the mid-exponential phase (Fig. 1). Recovery from growth inhibition by DCA was observed in succinate-utilizing cultures about 24 h after addition of the inhibitor, and culture absorbances subsequently increased to equal those measured in control flasks at the maximum-population-growth phase. No recovery from MCA inhibition was observed. Induction of dehalogenase activity in inhibited cultures was demonstrated by chloride ion release and by enzyme assays of cell extracts. Decrease in the pH of inhibited cultures was not a significant factor in accounting for inhibition, nor were glycolate, glyoxylate (products from dehalogenation of MCA and DCA, respectively), or chloride inhibitory at concentrations up to 100 mM. Neither MCA nor DCA inhibited the growth of succinate-utilizing *P. putida* PP1.

It was found that the extent of inhibition of *P. putida* strains by bromoalkanoates varied according to the structure and concentration of inhibitor and the dehalogenase phenotype of the strain under investigation. The most powerful inhibitor tested was monobromoacetate (MBA), which inhibited growth of succinate-utilizing (dehalogenase not induced) and 2MCPA-utilizing (dehalogenase induced) cultures of strain PP3 (Fig. 2A). In both cases, inhibition occurred instantaneously even at the lowest concentration of MBA that was tested (about 4 mM). *P. putida* PP1, the parent of strain PP3 which produced extremely low levels of dehalogenase activity (20), was also inhibited by MBA when utilizing succinate as the sole carbon and energy source. Other brominated alkanoates, 2MBPA and 2-monobromobutanoate (2MBBA), were inhibitory only to cultures of strain PP3 induced for dehalogenase activity with 2MCPA (Fig. 2B and 2C). At low concentrations of added inhibitor (2 to 3 mM), growth inhibition was either transient (2MBBA) or not detected (2MBPA). However, a marked and irreversible inhibition of the growth of strain PP3 was observed when 10 to 13 mM 2MBPA or 2MBBA was added to 2MCPA-utilizing cultures during the mid-exponential-growth phase. Brominated alkanoates were poor inducers of dehalogenase activity when compared with chlorinated analogs. However, dehalogenase activity with MBA, 2MBPA, and 2MBBA in cell extracts of strain PP3 was found to be higher than with MCA, 2MCPA, and 2MCBA, respectively (activity ratio, MCA:2MCPA:2MCBA:MBA:2MBPA:2MBBA was measured as 1:0.38:0.12:1.70:0.98:0.70).

Association between dehalogenase gene expression and growth inhibition by enzyme substrates was observed during studies of 2MCPA and 2MCBA utilization by mutants of strain PP3 producing elevated levels of dehalogenases. The mutants were isolated from chemostat cultures as described in Materials and Methods. Strain PP309 produced an elevated level of only dehalogenase I which was about 10-fold greater than that of *P. putida* PP3, whereas in strain PP3(R68.44) the specific activities of both dehalogenases were about four fold greater than those in strain PP3 (21; Weightman, Ph.D. thesis). The growth rates of both mutants were significantly lower than that of strain PP3 when growing on concentrations of 2MCPA higher than 0.3 g of carbon liter^{-1} (8.3 mM). Succinate-utilizing closed cultures of mutants inoculated directly from the chemostat were inhibited by additions of 2MCBA greater than 5 mM, whereas growth of *P. putida* PP3 was unaffected by additions of up to 20 mM 2MCBA or 2MCPA.

Resistance to growth inhibition by DCA and MCA in *P. putida* PP3. Mutants resistant to inhibition by DCA and MCA (DCA^r or MCA^r) were selected at high frequencies by plating overnight closed cultures of *P. putida* PP3 onto solid

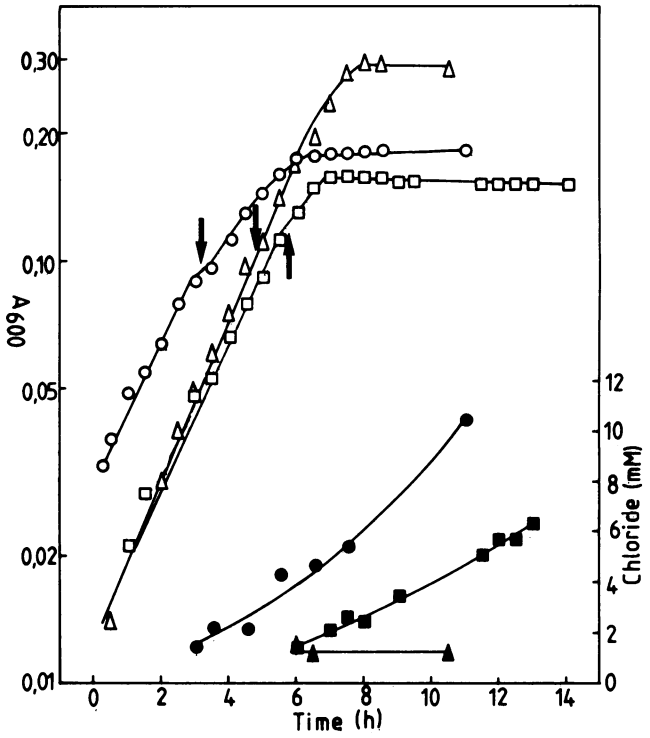
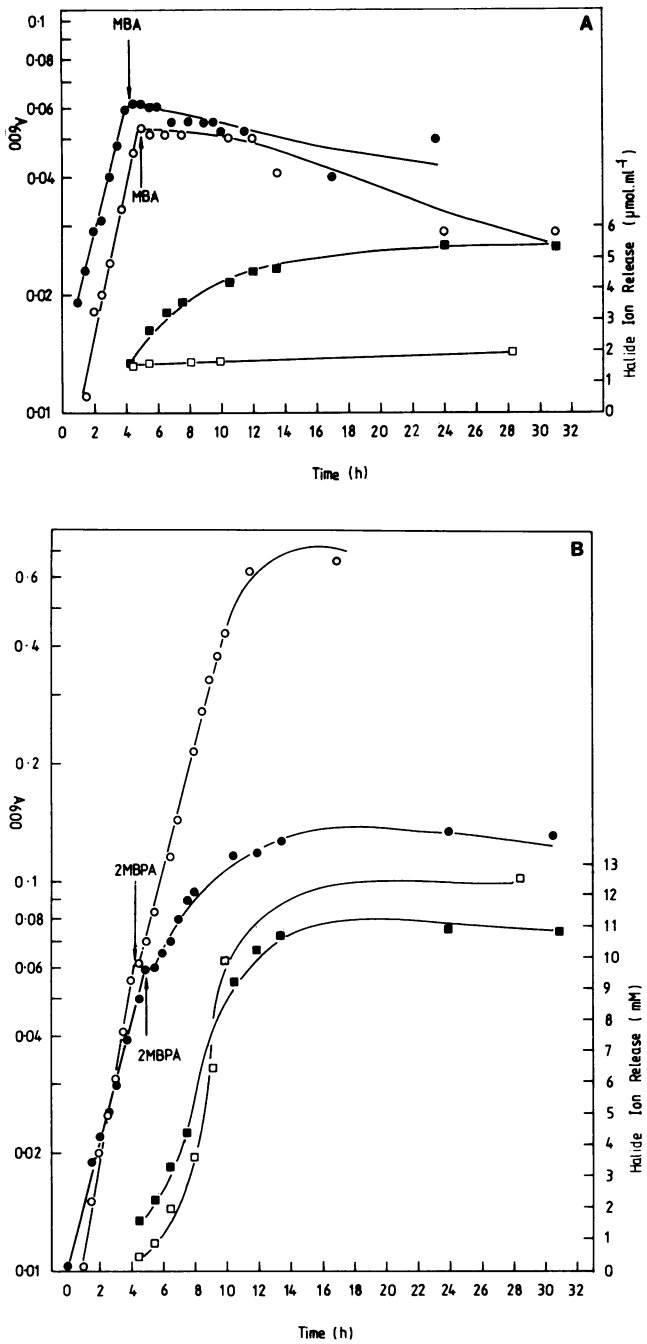


FIG. 1. Growth inhibition of *P. putida* PP3 by MCA and DCA added to succinate-utilizing closed cultures. Growth was monitored by measuring the culture A_{600} (○, □, △), and chloride ion release into the medium (●, ■, ▲) was estimated after additions (arrows) of 20 mM MCA–1 mM NaCl (○, ●), 20 mM DCA–1 mM NaCl (□, ■), or 1 mM NaCl (△, ▲). NaCl was added as a background to allow accurate chloride ion titration.

defined media containing succinate and either DCA or MCA (Table 1). About 50% of the DCA^r mutants were unable to utilize 2MCPA as a growth substrate, and a higher proportion of MCA^r mutants were 2MCPA negative. All MCA^r mutants tested (>500) were resistant to DCA, and, conversely, more than 500 DCA^r mutants were tested and found to be MCA^r.

A total of 20 MCA^r-DCA^r strains were selected for further study after isolation and purification on succinate-plus-DCA agar. Four classes of mutants were distinguished among these resistant strains. One class, PP40 mutants, included over half of the total MCA^r-DCA^r phenotype and could be easily identified by the inability of the mutants to utilize 2MCPA or 22DCPA as the sole carbon and energy sources. The PP40 mutants did not produce either dehalogenase I or II when grown in the presence of inducer (2MCPA), a result consistent with their inability to grow on dehalogenase substrates. About 70% of the mutants in this class were apparently unable to revert to 2MCPA and 22DCPA utilization at detectable frequencies (i.e., $<2 \times 10^{-11}$) despite having been isolated at a frequency of about 10^{-4}. However, five revertable mutants were isolated, with the frequencies of reversion varying from 2.1×10^{-3} to 1.8×10^{-7}.

The other three classes of DCA^r mutants (PP411, PP412, PP42) were all able to grow on 2MCPA and 22DCPA. PP42 mutants induced both dehalogenases and had the highest maximum growth rate (μ_{max}) on 2MCPA (Table 2) which corresponded to 80% of the maximum specific growth rate of strain PP3 on 2MCPA (0.34 h^{-1}). PP411 mutants induced only dehalogenase I and had higher maximum specific growth



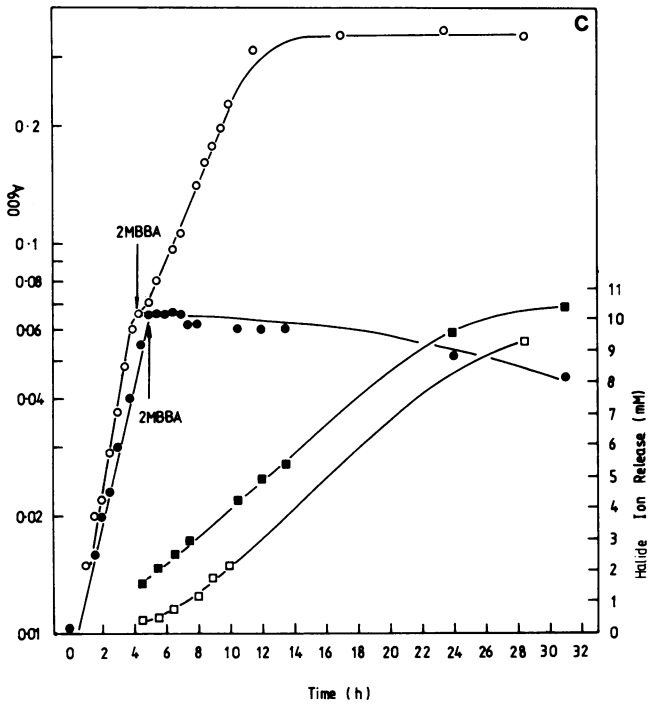


FIG. 2. Effects of the addition of MBA (A), 2MBPA (B), and 2MBBA (C) to closed cultures of *P. putida* PP3 growing on 2MCPA or succinate. Growth was monitored by measuring the culture A_{600} (○, ●), and halide ion concentration in the medium (□, ■) was monitored by titrations. Brominated alkanoic acids were added at a final concentration of 0.5 g of carbon liter^{-1} (20 mM MBA, 14 mM 2MBPA, 10.5 mM 2MBBA) to succinate-utilizing (○, □) and 2MCPA-utilizing (●, ■) cultures (arrows).

rates than the PP412 mutants which induced only dehalogenase II (Table 2). The difference in growth rates on 2MCPA of these three mutant classes was reflected in the dehalogenase specific activities induced by this substrate in three strains chosen as representatives of each class (Table 3).

Correlation between dehalogenase specific activities and observed μ_{max} values confirmed that the dehalogenase was the growth rate-limiting enzyme during catabolism of 2MCPA.

Only one class of mutants (PP5) was isolated from the enrichment for dehalogenase-negative strains by the method of Ornston et al. (16) (see above). After three cycles of antibiotic treatment, PP5 mutants unable to utilize 2MCPA and 22DCPA as growth substrates were isolated at a frequency of about 5×10^{-2}. All the mutants tested from this group were apparently nonrevertable (frequency of reversion to utilization of 2MCPA-22DCPA, $<2 \times 10^{-11}$) and were resistant to growth inhibition by MCA and DCA. Dehalogenase activity was not detected in PP5 mutants even after growth in the presence of 2MCPA.

Isolation of mutants capable of utilizing MCA as the sole carbon and energy source. The observed growth inhibition of *P. putida* PP3 by chlorinated acetates and the inability of this strain or the parent strain *P. putida* PP1 to grow on either glycolate or glyoxylate led us to recognize that at least two types of mutation would be required for the utilization of MCA and DCA as carbon and energy sources by *P. putida* PP3: a functional catabolic pathway for dissimilation of dehalogenase products had to be present, and inhibition by the substrates had to be alleviated.

Separate additions of MCA, DCA, glyoxylate, and glycolate at a final concentration of 5 mM to cell suspensions of *P. putida* PP3 did not stimulate respiration (oxygen uptake) above the observed endogenous rates. Spontaneous mutants of strains PP1 and PP3 able to grow on glyoxylate were isolated at a frequency of about 10^{-8}, and one such mutant from each strain was used to obtain spontaneous glycolate-utilizing mutants. No glycolate utilizers were isolated directly from the parent strains. A mutant growing on glycolate derived from *P. putida* PP3, designated strain PP301, utilized this substrate and glyoxylate as the sole carbon and energy sources (μ_{max}, 0.04 and 0.23 h^{-1}, respectively, in closed culture). The faster-growing strain PP3011 (μ_{max} on glycolate, 0.25 h^{-1}) was isolated from a chemostat culture initially inoculated with strain PP301, with glycolate sup-

plied as the sole growth-limiting carbon substrate (D = 0.075 h^{-1}). This mutant, isolated after 250 h of chemostat growth, had the same dehalogenase phenotype as *P. putida* PP3 and was unable to grow on MCA or DCA.

After establishing a glycolate-limited steady-state continuous-flow culture of strain PP3011 at a dilution rate of 0.075 h^{-1}, MCA (0.1 g of carbon $liter^{-1}$, 4.2 mM) was introduced into the medium reservoir, and the flow rate was adjusted so that the concentration of MCA in the culture vessel did not increase to inhibitory levels. Under these conditions, MCA induced dehalogenase activity in strain PP3011. Subsequently, the MCA concentration in the growth medium reservoir was increased to 0.5 g of carbon $liter^{-1}$, and simultaneously, the glycolate concentration was decreased until there was none left, and a steady-state culture was established which utilized MCA as the sole carbon and energy source. In this way the chemostat was used to select a population of MCA-utilizing organisms which were subsequently isolated from the chemostat culture on solid defined medium containing MCA at 0.5 g of carbon $liter^{-1}$. The dehalogenase activity of one MCA-utilizing mutant, strain PP3012, was measured in both continuous-flow and closed cultures (Table 4). The lower dehalogenase specific activities observed in closed cultures growing on MCA compared with those measured in chemostat culture were consistent with previously reported results (20, 21). In both types of culture, dehalogenase I and II were inducible since no significant activity was detected in succinate-grown cultures of strain PP3012.

Isolation of mutants utilizing DCA as the sole carbon and energy source. An MCA-limited steady-state culture of strain PP3012 was established in the chemostat (D = 0.075 h^{-1}), and a method similar to that outlined above was used to select for DCA-utilizing mutants. The DCA concentration in the medium reservoir was gradually increased and the MCA concentration was decreased until a steady-state culture utilizing DCA as the sole carbon and energy source was obtained (Table 4).

During 750 h of continuous-flow culture on DCA at a dilution rate of 0.075 h^{-1}, the observed biomass concentration remained low compared with values obtained in closed cultures inoculated from the chemostat and utilizing glyoxylate at an equivalent carbon concentration (0.2 g of carbon $liter^{-1}$). In addition, chloride ion release into the culture medium indicated that only 60% of the organically bound chlorine was mineralized, and so the steady-state culture contained a relatively high concentration of DCA. Growth of organisms taken from the culture and inoculated into liquidmedium containing DCA (0.2 to 0.5 g of carbon $liter^{-1}$) was very slow, and final biomass yields were low.However, because strain PP3012 failed to produce any growth on medium containing DCA, it was concluded that a DCA-utilizing mutant, designated strain PP3013, had been selected in the chemostat culture. At dilution rates below 0.05 h^{-1} and above 0.10 h^{-1}, washout of the DCA-utilizing culture occurred, indicating that the mutant designated PP3013 was poorly adapted for growth on DCA.

Dehalogenases I and II were detected in samples taken from the chemostat culture, although assays indicated induction of relatively low specific activities (Table 4). As with PP3012, dehalogenase activity in the DCA-utilizing strain was inducible.

TABLE 1. Frequency of *P. putida* PP3 mutation to DCA and MCA resistance

Selection medium	Frequency of isolation of DCA^r-MCA^r mutants	% Resistant mutants unable to grow on 2MCPA[a]
Succinate (0.5 g of C $liter^{-1}$) + DCA (1.0 g of C $liter^{-1}$)	6.0×10^{-4}	48
Succinate (0.5 g of C $liter^{-1}$) + MCA (1.0 g of C $liter^{-1}$)	3.9×10^{-2}	84
Succinate (0.5 g of C $liter^{-1}$) + MCA (1.5 g of C $liter^{-1}$)	2.6×10^{-2}	82

[a] >1,000 DCA^r mutants and >250 MCA^r mutants were tested on solid defined medium containing 2MCPA.

DISCUSSION

The growth inhibition of *P. putida* PP3 and related strains described in this paper was a direct effect of the halogenated alkanoates under investigation and probably resulted from the intracellular accumulation of inhibitory concentrations which, we suggest, was regulated by dehalogenase activities. This conclusion is supported by two observations. First, growth inhibition was shown not to be associated with catabolism of haloalkanoate dehalogenation products; nor was it due to a generalized pH effect. Second, the MICs of haloalkanoates for *P. putida* PP3 and other strains investigated in this study were found to vary widely depending on the levels of dehalogenase induced. Thus, strain PP1, the

TABLE 2. Variations in *P. putida* PP41 and PP42 phenotypes: dehalogenase activity ratios and growth rates when utilizing 2MCPA and lactate as sole source of carbon and energy

Phenotype[a]	Class	No. of mutants[b]	Dehalogenase activity ratio[c]		μ_{max} on 2MCPA (h^{-1})[d]	μ_{max} on lactate (h^{-1})
			MCA	DCA		
$2MCPA^+$ DCA^r MCA^r $dehI^+$ $dehII^-$	PP411	12	1	0.15 ± 0.03	0.22	0.45
$2MCPA^+$ DCA^r MCA^r $dehI^-$ $dehII^+$	PP412	3	1	1.62 ± 0.18	0.18	0.42
$2MCPA^+$ DCA^r MCA^r $dehI^+$ $dehII^+$	PP42	5	1	1.13 ± 0.21	0.24	0.44

[a] $2MCPA^+$, Utilization of 2MCPA and 22DCPA as growth substrates; dehI, expression or nonexpression of dehalogenase I when induced; dehII, expression or nonexpression of dehalogenase II when induced.

[b] Mutants were assigned to a class on the basis of results from disc-polyacrylamide gel electrophoresis of cell extracts and visualization of dehalogenase enzymes expressed in each strain.

[c] Calculated from assays with cell extracts. Arithmetical mean values are given with sample standard deviations (σ_{n-1}) calculated from dehalogenase specific activities obtained with each mutant in the appropriate class.

[d] μ_{max} was measured in closed culture with representative organisms from each class as follows: PP411-006, PP412-019, PP42-004.

TABLE 3. Dehalogenase activities in cell extracts of *P. putida* strains PP411-006, PP412-019, and PP42-004

Organism	Dehalogenase sp act (U mg of protein^{-1})[a]			
	MCA	DCA	2MCPA	22DCPA
PP411-006	0.112 ± 0.029	0.013 ± 0.002	0.047 ± 0.012	0.024 ± 0.007
PP412-019	0.152 ± 0.011	0.272 ± 0.020	0.019 ± 0.004	0.009 ± 0.001
PP42-004	0.241 ± 0.044	0.243 ± 0.077	0.082 ± 0.012	0.035 ± 0.008

[a] Dehalogenase activity ratios for MCA:DCA:2MCPA:22DCPA are as follows: 1:0.12:0.42:0.21 for PP411-006, 1:1.79:0.13:0.06 for PP412-019, and 1:1.01:0.34:0.15 for PP42-004. Standard deviations from arithmetic mean values of dehalogenase specific activities were calculated from enzyme assay data with three different cell extracts for each organism.

dehalogenase-negative parent strain of PP3 (19, 20), was unaffected by MCA and DCA, whereas hyperproducing dehalogenase strains such as PP309 were sensitive even to 2MCPA and 2MCBA substrates which had no significant effect upon the growth of strain PP3. The observed pattern of inhibition can be explained on the basis of a simple permease model in which genes encoding haloalkanoate transport proteins, as well as genes which encode the dehalogenases, are required by *P. putida* for growth on these substrates. Preliminary evidence for dehalogenase-associated permeases has been obtained in our laboratory from experiments designed to examine [1-^{14}C]MCA transport by some of the strains described in this study (J. H. Slater, A. J. Weightman, and B. G. Hall, submitted for publication). Further evidence of associations between dehalogenase gene expression and growth inhibition obtained from our study of the effects of brominated alkanoates upon the growth of induced and uninduced cultures of strain PP3 may be considered with regard to such a permease model. MBA was exceptional in that it was found to inhibit both PP1 and PP3 (whether induced or not), indicating that MBA is more generally toxic to *P. putida* than is its chlorinated analog MCA. However, 2MBPA and 2MBBA inhibited only the growth of cultures in which the dehalogenase system (dehalogenases, permeases) was induced. We suggest that differences between chlorinated and brominated alkanoates as substrates and inducers of the dehalogenase system account for these findings. Thus, 2MBPA and 2MBBA may be accumulated to toxic levels intracellularly by strain PP3 when the dehalogenase system is fully induced with 2MCPA. This effect is not observed with 2MBPA or 2MBBA alone, presumably because of the lower levels of permease(s) and dehalogenases induced by these substrates. An alternative explanation might account in part for our observations. 2MBPA and 2MBBA could act as competitive inhibitors of 2MCPA transport or dehalogenation, or both, reducing the rate of 2MCPA catabolism to levels which would not support growth. However, because 2MBPA is a good growth substrate for strain PP3, it seems unlikely that it would competitively inhibit growth on 2MCPA. We have not determined the mechanism of growth inhibition, although the inhibitory effects of various halogenated alkanoic acids have been widely reported, indicating that several possible mechanisms exist (2, 4, 17, 18).

To our knowledge, there is only one other report of the inhibitory effects of haloalkanoates on dehalogenase-producing microorganisms. Allison et al. (N. Allison, A. J. Skinner, and R. A. Cooper, Abstr. 2nd Intl. Symp. Microb. Ecol. 1980, no. 16-5) reported that growth of *Rhizobium* sp. was inhibited by MCA and DCA, but they did not attempt to isolate resistant mutants.

Two independent methods of selection resulted in the isolation of MCA-DCA-resistant strains from *P. putida* PP3. Mutations giving the resistant phenotype were clearly associated with genes encoding enzymes of the dehalogenase system because MCA^r-DCA^r mutants (i.e., PP411, PP412, PP42, and PP40) selected solely by resistance to chlorinated acetates all showed altered patterns of dehalogenase induction by 2MCPA. Conversely, selection of PP5 mutants from strain PP3 on the basis of an inability to utilize 2MCPA as a carbon and energy source resulted in the coselection of the MCA^r-DCA^r phenotype. We conclude that catabolism of dehalogenase products such as lactate was unaffected in all mutants examined because μ_{max} values obtained were not significantly different from that of the parent strain PP3. We propose that expression of resistance phenotypes results from modulation of transport of inhibitory haloalkanoate substrates which in turn may be affected by mutations of the gene(s) encoding permeases for these substrates. The variety of dehalogenase phenotypes associated with MCA-DCA

TABLE 4. Dehalogenase activities of *P. putida* PP3012 and PP3013 in continuous-flow and closed cultures

Culture	Dehalogenase sp act (U mg of protein^{-1})[a]				
	MCA	DCA	2MCPA	22DCPA	2MCBA
PP3012					
Continuous flow; substrate, MCA (D = 0.1 h^{-1})	0.65 (1)	0.90 (1.38)	0.21 (0.32)	0.10 (0.15)	ND
Continuous flow; substrate, MCA (D = 0.05 h^{-1})	0.40 (1)	0.70 (1.75)	0.10 (0.25)	0.06 (0.15)	0.03 (0.08)
Closed; substrate, MCA	0.18 (1)	0.31 (1.72)	ND	ND	ND
PP3013 (continuous flow; substrate, DCA [D = 0.06 h^{-1}])	0.53 (1)	0.46 (0.87)	0.09 (0.17)	0.05 (0.09)	ND
PP3					
Continuous flow; substrate, 2MCPA	0.41 (1)	0.39 (0.95)	0.11 (0.27)	0.06 (0.15)	ND
Closed; substrate, 2MCPA	0.12 (1)	0.18 (0.86)	0.07 (0.33)	0.03 (0.14)	0.02 (0.10)

[a] Activity ratios relative to MCA are given in parentheses. ND, Not determined.

resistance, apparent from the results presented in this paper, probably reflects genotypic variation among mutant classes. In general, resistant strains are marked either by a reduction in levels of dehalogenase(s) or by complete loss of activity, whereas dehalogenase-hyperproducing strains selected in chemostat culture (21; Weightman, Ph.D. thesis) are inhibited even by the less toxic haloalkanoates such as 2MCPA. It is of interest to note that all dehalogenase-producing mutants of strain PP3 retain regulated expression of dehalogenase(s). We have already reported strong evidence for the close genetic linkage between and coordinate control of dehalogenase-associated functions (1, 20). It seems reasonable to suggest, therefore, that mutations in a permease gene(s) which result in increased or decreased tolerance to haloalkanoate inhibitors may also affect dehalogenase gene expression and that mutations in dehalogenase genes may through polarity effects or if they are deletions alter or abolish permease gene expression.

Another important class of DCA^r-MCA^r mutants, designated PP4120, has recently been identified and found to be most closely related to PP412 mutants in that only dehalogenase II is induced (Slater et al., submitted for publication). In PP4120 mutants the resistance phenotype is associated with extremely low growth rates on 2MCPA. The only satisfactory explanation for this phenotype, in view of the finding that PP4120 and PP412 mutants express similar dehalogenase-specific activities when induced with 2MCPA, is that PP4120 mutants cannot transport the substrate at a rate commensurate with good growth and that they are, therefore, permease mutants.

The utilization of MCA and DCA as carbon and energy sources required several mutations in strain PP3. Our observation that glycolate-utilizing mutants were not isolated directly from strain PP3 but only from glyoxylate utilizers could be explained if the catabolism of both substrates proceeded via the tartonic semialdehyde pathway (6) by which glycolate is first converted by dehydrogenation to glyoxylate and then to malate via several intermediates. The inability of glyoxylate-utilizing mutants of strain PP3 to utilize glycolate as the sole carbon and energy source indicated that at least two mutations were required to produce the PP301 phenotype. MCA- and MCA-DCA-utilizing strains, PP3012 and PP3013, respectively, were resistant to growth inhibition by MCA and DCA when growing on succinate. In this respect, these strains were phenotypically similar to PP42 mutants, inducing both dehalogenase I and II. The low growth yield and slow growth of strain PP3013 on DCA compared with growth of the mutant on glyoxylate (the dehalogenated product) indicated that inhibition by the substrate was not fully overcome despite prolonged continuous-flow culture of strain PP3013 with DCA as the growth-limiting substrate.

An important finding in this work is that most of the dehalogenase-negative mutants (PP40 and PP5) did not revert at detectable frequencies to reacquire the ability to utilize 2MCPA-22DCPA as a growth substrate. These mutants were selected at high frequencies (about 10^{-4}), strongly suggesting that their mutations involved deletions of DNA associated with genes encoding enzymes of the dehalogenase system in strain PP3. Thus, the genes may have either been lost or become cryptic. High deletion frequencies could be explained if transposable genetic elements are involved in dehalogenase gene regulation or, indeed, if dehalogenases and associated functions are encoded by transposons. However, there is no direct evidence for dehalogenase transposons. Alternatively, there is the possibility that the genes are carried on a plasmid. In the case of *P. putida* PP3, we have no indication that dehalogenase genes are plasmid borne. Plasmid DNA has never been detected in this strain, despite the use of several techniques designed to isolate large and unstable plasmids (J. R. Beeching, P. C. Gowland, and J. H. Slater, unpublished observations), nor can dehalogenase activity be transferred directly from strain PP3 to other pseudomonads.

The mutants described in this paper enabled us to examine different elements of the dehalogenase system of strain PP3 for the first time and presented some important questions with respect to the evolution of this organism in the microbial community from which it was isolated. For example, does instability of the dehalogenase system reflect the way in which strain PP1 mutated to produce strain PP3? The isolation of mutants which do not express either one or the other of the dehalogenase genes (PP411 and PP412) but which are still able to grow on 2MCPA demonstrates that both dehalogenase enzymes are not necessarily required for selection of this phenotype. Yet selection of strain PP3 as a mutant of strain PP1 in continuous-flow culture produced a strain which coordinately induces two mechanistically unrelated (and, therefore, probably evolutionarily unrelated) enzymes (5, 19, 23).

Furthermore, the significance in the biosphere of a mutation which, on the one hand, allows utilization of novel substrates for growth, but which also makes the strain susceptible to inhibition by related substrates, is difficult to determine. The results from this study do, however, illustrate some important points with regard to the selection of mutant strains capable of utilizing novel substrates. Four factors have now been recognized that determine the utilization by strain PP3 of dehalogenase substrates as the sole carbon and energy sources: (i) toxicity of the substrate; (ii) presence of catabolic pathways for the dissimilation of dehalogenated products; (iii) induction of dehalogenase and permease at levels allowing growth but not resulting in the accumulation of inhibitory concentrations; and (iv) activity of either one or both enzymes at rates commensurate with growth on the substrate.

ACKNOWLEDGMENTS

We thank Shell Grants Committee for an unrestricted grant for part of this work. J.H.S. acknowledges support from the Natural Environment Research Council.

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