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Dehalogenase from *Methylobacterium* sp. HJ1 induced by the herbicide 2, 2-dichloropropionate (Dalapon)

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Heavy industrial activities and agricultural processes require consumption of many halogenated compounds, and release them continuously as pollutants into the environment. These xenobiotics show high toxicity and persistence and cause many problems to the society, soils and ground water. Microbial dehalogenases are involved in the biodegradation of many important chlorinated compounds. A bacterial strain identified as *Methylobacterium* sp. HJ1 is able to degrade the herbicide 2,2-dichloropropionic acid by removal of the halogen and subsequent metabolism of the product for energy. D,L-2-chloropropionate also supported good growth of the organism but 3-chloropropionate, monochloroacetate and dichloroacetate were not utilized. Cell-free extracts of the 2,2-dichloropropionate-grown bacteria converted 2,2-dichloropropionate into pyruvate with the release of two chloride ions for each molecule of pyruvate formed. This indicates the presence of dehalogenase activity in the cell-free extracts. Only 2,2-dichloropropionate and D,L-2-chloropropionate were inducers and substrates for the dehalogenase. Monochloroacetate and dichloroacetate did not serve as an inducer, whereas 3-chloropropionate was a non-substrate inducer.

Key words: Dehalogenase, 2,2-dichloropropionic acid, degradation, *Methylobacterium* sp.

INTRODUCTION

Synthetic halogenated organic compounds are found widely throughout the biosphere due to high consumption in modern industrial and agricultural processes. These xenobiotic compounds show high toxicity and persistence, and cause the contamination of soils and ground waters. The microbial catabolism of these compounds has been reviewed extensively (Hardman, 1991; Leisinger and Bader, 1993) . The herbicide 2,2-dichloropropionic acid can be used to control specific annual and perennial grasses, such as quick grass, Bermuda grass and cattails. It is an effective inhibitor of panothenic acid production (Prasad and Blackman, 1965) and pyruvate utilization in microorganisms (Redemann and Meikle, 1955). Microorganisms can be adapted to metabolize monochlorinated and dichlorinated aliphatic acids (Hirsch and Alexander, 1960; Olaniran et al., 2001). Certain species of soil microorganism can readily attack 2,2-dichloropropionic acid and use it as a carbon source with the rapid liberation of chloride ions. In this paper, the properties of a newly isolated bacterium from agricultural soil in Malaysia that is able to utilize 2,2-dichloropropionate as the sole source of carbon and energy will be discussed.

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MATERIALS AND METHODS

Growth media preparation

Bacterial cells were grown in PJC chloride-free minimal media. A stock solution for PJC media was prepared as a ten-fold concentration of basal salts containing $K_2HPO_4.3H_2O$ (42.5 g/l), $NaH_2PO_4.2H_2O$ (10.0 g/l) and $(NH_4)_2SO_4$ (25.0 g/l). The trace metal salts solution was a ten-fold concentrated stock that contained nitriloacetic acid $C_6H_9NO_6$ (1.0 g/l), $MgSO_4$ (2.0 g/l), $FeSO_4.7H_2O$ (120.0 mg/l), $MnSO_4.4H_2O$ (30.0 mg/l), $ZnSO_4.H_2O$ (30 mg/l) and $CoCl_2.6H_2O$ (10 mg/l) in distilled water (Hareland et al., 1975). Minimal media for growing bacteria contained 10 ml of ten-fold basal salts and 10 ml of ten-fold trace metal salts per 100 ml of

distilled water, and were autoclaved (121°C for 15 min at 15 psi).

Carbon sources (1 M 2,2-dichloropropionate) was filter-sterilised (0.2 m pore size) separately and added aseptically to the media to the desired final concentration. In order to prepare solid medium, Oxoid bacteriological agar (1.5% w/v) was added prior to sterilisation. Samples were removed periodically and the growth was determined by measuring the turbidity at A_{680nm}.

Preparation of cell-free extracts

Cell-free extracts were prepared from cells grown in PJC minimal media supplemented with 20 mM 2,2- dichloropropionate and used for enzyme assay analysis. Cells were harvested at late logarithmic phase and washed twice with ice-cold distilled water to remove any excess chloride ions. The cells were resuspended in 0.1 volume of 0.1 M Tris phosphate, 1 mM EDTA, 10% (w/v) glycerol, pH 7.2, and ruptured by ultrasonication (Vibra Cells^{TM}) for 5 min. Sonicated cells were centrifuged at 10,000 g for 10 min and the cell debris discarded. All manipulations of the cell-free extracts were carried out at 0^{O}C. Protein concentration was determined by the biuret procedure with serum albumin as a standard (Gornall et al., 1949).

16S rRNA gene analysis

PCR amplification of the 16S rRNA gene for bacterial identification was carried out using the colony PCR technique. The primers used were forward primer, FD1 (5'-aga gtt tga tcc tgg ctc ag-3') and reverse primer, rP1 (5'-acg gtc ata cct tgt tac gac tt-3'), based on conserved regions of the 16S rRNA gene (Sander et al., 1996). The sequence obtained was blasted in the EMBL database using the FASTA 3 tool (Pearson and Lipman, 1988). The best match obtained was to *Methylobacterium* sp. The final gene sequence was submitted to the EMBL GeneBank under accession number AM231910.

Pyruvate estimation

Pyruvate was estimated colorimetrically according to Friedman and Haugen (1943), assuming the molar absorption coefficient of alkaline pyruvate 2,4-dinitrophenylhydrazone to be 18 000 at A_{445nm} (Kornberg and Morris, 1965). Pyruvate formation from 2,2-dichloropropionate by cell-free extracts was followed continuously at A_{315nm} and 30^{O}C using a Jenway 6300 series recording spectrophotometer. The assay mixture contained: 100 mol 2,2-dichloropropionate; 1.74 mg phenylhydrazine hydrochloride; 0.48 ml RIM buffer. The RIM buffer contained: 200 mol EDTA, pH 6.8; 100 mol $MgCl_2$; 500 mol imidazole buffer, pH 6.8; and distilled water to a final volume of 4 ml. The reaction was started by the addition of cell-free extract containing 100 - 800 g soluble protein to the test cuvette. Specific activities were calculated as mol pyruvate formed min^{-1} (mg protein)$^{-1}$.

Chloride ion assay

Cell- free extracts were assayed for the ability to release chloride ions in a mixture containing the extract (2 - 3 mg protein), 1 ml of 0.2 M phosphate buffer pH 6.8, and 2,2- dichloropropionate as substrate (final concentration 1 mM) in a total volume of 5 ml. The reaction was initiated by adding the extract to the substrate and buffer and maintained in a water bath at 30°C for 20 min. The reaction was terminated by pipetting a 1-ml aliquot every 5 min into 100 l of 0.25 M ammonium ferric sulphate in 9 M nitric acid, followed by 100 l of mercuric thiocyanate for the measurement of chloride ion. Chloride ion released was measured at A_{460nm} (Iwasaki et al., 1956). Specific activity was defined as the amount of chloride (g) liberated per mg protein per minutes. All values were corrected against boiled enzyme controls, where no chloride liberation was noted.

RESULTS

Characterisation of a 2,2-dichloropropionate-degrading microorganism

A pure culture was isolated that demonstrate the ability to decompose 2,2-dichloropropionate. The isolate utilised a number of non-chlorinated compounds as carbon source including glucose, sucrose, lactate, pyruvate, mannitol and glycerol. However, it did not grow on 3-chloropropionate, monochloroacetate, dichloroacetate, glyoxylate, glycolate and malate. The generation time of the isolate in PJC medium supplied with 2,2- dichloropropionate as sole source of carbon was 14 h at 30^{O}C. The addition of yeast extract at a final concentration of 0.05% (w/v) reduced the doubling time with 2,2-dichloropropionate to 5 h at 30^{O}C. The cell doubling-times in mineral salts medium with pyruvate, lactate and glucose as sole sources of carbon were 1.8, 2.8 and 3.2 h, respectively, at 30^{O}C. The isolate did not grow at 5 or 37^{O}C.

The organism is a Gram-negative rod-shaped bacterium and forms a pinkish color in PJC minimal medium with 2,2-dichloropropionate as the sole source of carbon. Cells from young cultures were motile. Colonies, which were 1 to 4 mm in diameter after 4 days at 30^{O}C, had a rough surface, a smooth margin and a raised elevation. The cells were acid-fast and did not produce spores. The bacterium also utilize lactose, liquefies gelatin and produces catalase and oxidase. These results suggest that the 2,2-dichloropropionate utilizing isolate is *Methylobacterium*. In support of this view, the 16S rRNA technique was carried out and we have proposed classification of the bacterium as *Methylobacterium* sp. based on over the 1300 bases of the area sequenced with an identity of 99.7% with the published sequence. The nucleotide sequence appear in EMBL data base with the accession number AM231910. It was selected for further studies because of its ability to utilise 2,2-dichloro-propionate at higher rate of the chlorinated aliphatic acid.

Pyruvate production from 2,2-dichloropropionate by cell-free extracts

The enzyme in cell-free extracts readily converted this compound to pyruvate (Table 1). There was no pyruvate formation when D,L-2-chloropropionate or chloroacetate replaced 2,2-dichloropropionate in the assay mixture. Extracts of bacteria grown on D,L-2-chloropropionate was also able to convert 2,2-dichloropropionate to pyruvate but only at one-eighth of the rate observed using extracts from 2,2-dichloropropionate grown cells (Table 1). Extracts of bacteria grown on lactate, pyruvate, mannitol, glycerol, and glucose did not form pyruvate from 2,2-

Table 1. Pyruvate formation from 2,2-dichloropropionate by cell-free extracts of bacteria grown on various carbon sources.

Carbon sources for growth	Pyruvate formed [mol min^{-1}(mg protein)$^{-1}$]
2,2-dichloropropionate	83
D,L-2-chloropropionate	12
Lactate	ND
Lactate + 2,2-dichloropropionate	50
Pyruvate	ND
Pyruvate + 2,2-dichloropropionate	19
Mannitol	ND
Mannitol + 2,2-dichloropropionate	15
Glycerol	ND
Glycerol + 2,2-dichloropropionate	12
Glucose	ND
Glucose + 2,2-dichloropropionate	14
Lactate + D,L-2-chloropropionate	7
Pyruvate + D,L-2-chloropropionate	10
Mannitol + D,L-2-chloropropionate	6
Glycerol + D,L-2-chloropropionate	7
Glucose + D,L-2-chloropropionate	10
Lactate + 3-chloropropionate	ND
Lactate + Monochloroacetate	ND
Lactate + Dichloroacetate	ND

Note: ND (not detected).

dichloropropionate. However, growth on these non-chlorinated compounds in the presence of 2,2-dichloropropionate resulted in induction of the pyruvate forming system (Table 1). Extracts prepared from cells grown on lactate in the presence of 3-chloropropionate, monochloroacetate and dichloroacetate did not convert 2,2-dichloropropionate to pyruvate.

Chloride ion release by cell-free extracts

Chloride ion release and pyruvate formation from 2,2-dichloropropionate is expected to accompany the formation of pyruvate. Cell-free extracts of the bacterium grown on 2,2-dichloropropionate as the sole source of carbon liberated chloride ions from this compound when added to an incubation mixture containing 0.2 M phosphate buffer, pH 6.8, and 2,2-dichloropropionate (Table 2). The specific activity of the dehalogenase was 12.9 molCl^-/min/mg protein. When samples of the incubation mixture were assayed for chloride ions and pyruvate (Friedemann and Haugen,. 1943), one molecule of the latter compound was present for each two chloride ions.

Chloride ions were also released from other chlorinated aliphatic acids (Table 2). The rate with D,L-2-chloropropionate was about four times higher than with 2,2-dichloropropionate. However, there was no chloride ion release from 3-chloropropionate. Chloride ion release from monochloroacetate was more rapid than from 2,2-dichloropropionate. Chloride ions were also liberated from dichloroacetate similar to the rate observed from 2,2-dichloropropionate. The Michaelis-Menten constants for 2,2-dichloropropionate, D,L-2-chloropropionate, monochloroacetate and dichloroacetate were 0.5, 1.2, 2.2 and 2.5 mM, respectively.

DISCUSSION

We have isolated one bacterial strain and identified it as *Methylobacterium* sp. HJ1 using biochemical and 16S rRNA analysis. The bacterium effectively dechlorinates 2,2-dichloropropionate. This is the first reported strain of *Methylobacterium* isolated from Malaysian agricultural soil that can be induced to synthesizes dehalogenase by 2,2-dichloropropionate. Species of *Methylobacterium* have not previously been reported to degrade 2,2-dichloropropionate but were mainly associated with chloromethane metabolism (Vanneli et al., 1998). Studies on microbial decomposition of 2,2-dichloropropionate have been reported earlier by Jensen (1960), Magee and Colmer (1959) and Senior et al. (1976). The formation of pyruvate from 2,2-dichloropropionate by extracts of the bacterium grown on this compound as sole source of carbon was readily demonstrated in agreement with the earlier observation of Kearney et al. (1965). Both chloride ions were released from 2,2-dichloropropionate for each molecule of pyruvate formed. The conversion of 2,2- di-

Table 2. Chloride ion release from chlorinated aliphatic acids by cell-free extracts of 2,2-dichloropropionate grown bacteria.

Substrate	Chloride ion released [(mol min^{-1}(mg protein)$^{-1}$]
2,2-dichloropropionate	10.2
D,L-2-chloropropionate	40.3
3-chloropropionate	ND
Monochloroacetate	35
Dichloroacetate	11

Note**:** ND (not detected)

propionate to pyruvate may be taken as a measure of dehalogenase activity in this bacterium. Both 2,2- dichloropropionate and 2-chloropropionate served as inducers of dehalogenase activity under the same conditions.

Studies on the substrate specificities of dehalogenases from 2,2-dichloropropionate- utilizing microorganisms have been reported earlier by Kearney et al. (1965) and Slater et al*.* (1976). Our study indicated that chloride ions were liberated from 2,2-dichloropropionate and D,L-2-chloropropionate with two chloride ions being released from 2,2-dichloropropionate compared with one from D,L-2-chloropropionate.

Dichloroacetate and monochloroacetate, which were also substrates for the dehalogenase, did not support growth of the isolate. In the case of dichloroacetate this may be due to the inability of the organism to utilize glyoxylate which is formed from dichloroacetate (Goldman, 1972). The inability of monochloroacetate to induce the dehalogenase could account for the failure to grow on the glycolate compound.

Dichloroacetate served as a substrate for the dehalogenase from an *Arthrobacter* sp. (Kearney et al*.*, 1965,) . The corresponding enzyme from *Pseudomonas putida* possessed activity towards dichloroacetate and monochloroacetate (Slater et al*.,* 1976), whereas, *Burkholderia cepacia* MBA4 possess activity towards the latter substrate only (Murdiyatmo et al., 1992). The 3-chloropropionate did not induce the dehalogenase and was not a substrate for the enzyme. The dehalogenase from *Arthrobacter* (Kearney et al., 1965) and *Rhizobium* sp. (Leigh et al., 1988) also showed no activity with 3-chloropropionate, whereas the enzyme from *P. putida* showed slight activity towards this substrate (Slater et al., 1976).

The present study has shown a species of *Methylobacterium* capable of growing on 2,2-dichloropropionate; readily converting it to pyruvate by means of an inducible dehalogenase. This enzyme showed activity with a variety of chlorinated aliphatic acids and can be induced by 2, 2-dichloropropionate and also by D,L-2-chloropropionate. Additional information on the proper-ties of the enzymes from cells grown under various conditions will lead to a greater understanding of the degradation of 2,2-dichloropropionate and related compounds.

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